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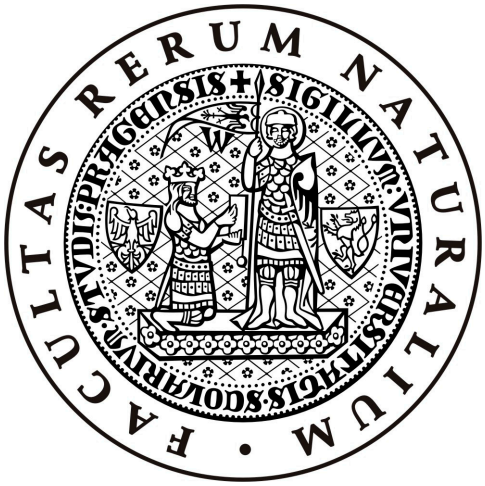


**Ing. Kateřina Vodičková Kepková**

# Transkriptomika embryonální genomové aktivace preimplantačního vývoje skotu v podmínkách *in vivo* a *in vitro* kultivace

# Transcriptomics of bovine preimplantation embryo genome activation *in vivo* and in *in vitro* culture conditions

**Disertační práce**

**Školitel: RNDr. Jiří Kaňka, DrSc.**

**Praha, 2011**

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V Praze,

Podpis

Disertační práci jsem vypracovala na Ústavu živočišné fyziologie a genetiky Akademie věd České republiky, v.v.i., v Liběchově.

## Poděkování

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# OBSAH

# ABSTRAKT

Cílem práce bylo charakterizovat transkripční profil *in vivo* a *in vitro* získaných embryí během bovinní minoritní a majoritní genomové aktivace a dále identifikovat mRNA transkripty, které jsou nově syntetizované během těchto stádií. V naší první studii jsme se zaměřili na studium minoritní aktivace genů ve 4-buněčném stádiu embrya. Pomocí metody supresivní subtraktivní hybridizace (SSH) jsme nalezli 31 amplikonů homologních s již známými geny. Pro podrobnější studium exprese během celého období preimplantačního vývoje jsme vybrali 5 genů: *centromere protein, 350/400 kDa (CENPF, mitosin), splicing factor arginine/serine-rich 3 (SRFS3), high mobility group nucleosomal binding domain 2 (HMGN2) protein* a eukaryotické translační iniciační faktory *EIF4A2* a *EIF4E*. Všechny tyto geny hrají důležitou roli v raném vývoji embrya. Gen *SRFS3* je prvním genem s významnou funkcí, jehož exprese byla nalezena již v průběhu minoritní genomové aktivace, což potvrdila citlivost jeho transkripce k α-amanitinu v tomto stádiu. Pro další studium jsme si vybrali gen *CENPF* (*centromeric protein F, mitosin*), jehož funkci během preimplantačního vývoje bovinního embrya jsme podrobně studovali pomocí specifického umlčení injikací double-stranded (dsRNA) do embrya ve stádiu zygoty. Zjistili jsme, že mikroinjekce *CENPF* dsRNA efektivně a specificky způsobuje degradaci mRNA *CENPF*. Při imunofluorescenčním značení pomocí anti-CENPF protilátky byla u *CENPF* dsRNA injikovaných embryí úroveň fluorescence velmi nízká oproti kontrolám a blastomery *CENPF* dsRNA injikovaných embryí měly často fragmentovaná nebo úplně chybějící jádra. Vývojová kompetence zaznamenala významný pokles, pouze 28,1% *CENPF* dsRNA injikovaných 8-buněčných embryí bylo schopno pokračovat ve vývoji do stádia 16 buněk. Tyto výsledky ukazují, že deplece *CENPF* mRNA vede k dramatickému poklesu vývojové kompetence bovinních embryí po embryonální genomové aktivaci. Při stanovení transkripčního profilu minoritní a majoritní genomové aktivace (ve stádiu 4 a 8 buněk) v *in vivo* získaných a *in vitro* kultivovaných embryích skotu jsme použily specifický preimplantační bovinní mikročip (BlueChip). Nejprve jsme pomocí sérií SSH připravili cDNA knihovny (4-buněčné stadium – MII oocyty, 4-buněčné stádiu – 8-buněčné stadium a 8-buněčné stadium – 4-buněčné stadium) a vybrané cDNA z těchto knihoven byly použity v návrhu verze 3 mikročipu BlueChip. Mezi 4-buněčnými *in vivo* a *in vitro* embryi bylo rozdílně exprimováno 134 genů a mezi 8-buněčnými embryi 97 genů. Pomocí metody qRT-PCR byl stanoven expresní profil během celého preimplantačního vývoje u 7 vybraných genů (*BUB3, CUL1, FBL, NOLC1, PCAF, GABPA* a *CNOT4*). Analýza odhalila významné rozdíly v hladině transkriptů mezi *in vitro* a *in vivo* získanými embryi v pozdních 8-buněčných stádiích u genů *BUB3, NOLC1, PCAF, GABPA* a *CNOT4*. Identifikovali jsme přepnutí genové exprese dvou příbuzných genů *cullin 1-like*, transkripční varianty 1 (UniGene ID BT.36789) na *cullin 1*, transkripční variantu 3 (UniGene ID BT.6490) během majoritní genomové aktivace ve stádiu 8 buněk. Pomocí imunofluorescence jsme nalezli nově syntetizovaný protein fibrillarin již ve stádiu raných 8 buněk po *in vitro* kultivaci. Tato detekce je potvrzena pomocí metody qRT-PCR na úrovni mRNA. Nalezené geny mohou sloužit jako ukazatel kvality embryí ve snaze optimalizovat podmínky kultivace *in vitro*.

## Abstrakt

The goal of the thesis was to characterize transcriptional profiles of *in vivo* and *in vitro* derived embryos during bovine minor and major embryonic genome activation and to identify mRNA transcripts newly synthesized during these stages. In our first work we have concentrated on the study of minor genome activation at the 4-cell stage of embryo. Using SSH, we have identified 31 amplicons homologous with already identified genes. We have selected 5 of these for detailed study of their expression during the whole period of preimplantation development: *centromere protein, 350/400 kDa (CENPF, mitosin), splicing factor arginine/ serine-rich 3 (SRFS3), high mobility group nucleosomal binding domain 2 (HMGN2) protein* and eukaryotic translation initiation factors *EIF4A2* a *EIF4E*. All these genes play an important role in the early embryo development. *SRFS3* is the first described gene with an important function in preimplantation development, which is expressed already during bovine minor genome activation, and its transcription is α-amanitin sensitive during this period. We have selected *CENPF* gene for a more thorough study. By silencing its expression by the injection of *CENPF* dsRNA into the zygote, we have studied its function throughout the whole preimplantation development of bovine embryo. Microinjection of *CENPF* dsRNAS resulted in specific and effective degradation of *CENPF* mRNA. Immunofluorescence staining with anti-CENPF antibody showed very low fluorescence levels in *CENPF* dsRNA injected embryos comparing to uninjected controls and blastomeres of *CENPF* dsRNA injected embryos often had fragmented or missing nuclei. There was apparent marked decrease in a developmental competence, only 28.1% of the *CENPF* dsRNA injected 8-cell stage embryos reached 16-cell stage. It can be concluded, that depletion of *CENPF* mRNA leads to dramatic decrease of developmental competence of bovine embryos at the stage of major genome activation. We have used bovine preimplantation embryo specific cDNA microarray (BlueChip) to characterize transcriptional profile of the *in vivo* derived and *in vitro* prepared bovine embryos at the stage of minor (4-cell stage) and major (8-cell stage) genome activation. First we have prepared SSH cDNA libraries (4-cell stage embryos – MII oocytes, 4-cell stage embryos – 8-cell stage embryos and 8-cell stage embryos – 4-cell stage embryos) and selected cDNAs from these libraries were used in BlueChip version 3 array design. We have identified 134 genes as differentially expressed between bovine 4-cell stage in vivo and in vitro embryos and 97 genes as differentially expressed between 8-cell stage in vivo and in vitro embryos. Using qRT-PCR, we have characterized expression profile of 7 selected genes (*BUB3, CUL1, FBL, NOLC1, PCAF, GABPA* and *CNOT4*) throughout preimplantation development. Our analysis revealed significant changes in the level of expression of *BUB3, NOLC1, PCAF, GABPA* and *CNOT4* at the late 8-cell stage. We have also identified switch in the expression of two related genes, from the *cullin 1-like*, transcript variant 1 (UniGene ID BT.36789) to *cullin 1*, transcript variant 3 (UniGene ID BT.6490) during the time of major genome activation at 8-cell stage. Using immunofluorescence staining, we have found fibrillarin protein already at the early 8-cell stage *in vitro* cultured embryos. This finding was also confirmed on mRNA level by qRT-PCR. The complex of these genes could be used as a tool for assessing embryo quality for optimization of IVP embryo culture conditions.

# 1. ÚVOD

Během raného embryonálního vývoje se vyskytuje mnoho kritických molekulárních událostí, mezi nejdůležitější patří aktivace embryonálního genomu a diferenciace buněk. Porozumění těmto událostem je důležité nejen pro základní biologii, ale i pro praktické aplikace zahrnující produkci hospodářských zvířat a aplikace v regenerativní medicíně (Ko, 2004). Bovinní a humánní preimplantační vývoj je velice podobný z hlediska načasování epigenetické reprogramace, aktivace embryonálního genomu a délkou preimplantačního vývoje, proto výsledky studií na *in vivo* bovinních embryích mohou být cenné pro hodnocení humánních embryí produkovaných rozdílnými technikami asistované reprodukce (ART). Rozvoj nových genomických technik, jako jsou mikročipy nám může poskytnout komplexní pohled na genovou expresi a dynamiku transkriptomu v raném embryonálním vývoji. Charakterizace normálních maternálních a embryonálních transkriptomů je důležitá pro porozumění embryogenezi a zlepšení technik asistované reprodukce (Kues et al., 2008).

*In vitro* fertilizace (IVF) je velice rozšířená technika. První úspěch v humánní medicíně slavila roku 1978 prvním narozeným dítětem (Steptoe a Edwards, 1978), které znamenalo revoluci v léčbě neplodnosti. Postupně se tato metodika začala zavádět i do veterinární praxe, kde může sloužit zejména k získání potomstva od geneticky cenných jedinců. První tele se po aspiraci *in vivo* maturovaných oocytů, *in vitro* oplození a následném přenosu do recipientky narodilo roku 1981 (Brackett et al., 1982). Od té doby vedl výzkum k vyvinutí systému *in vitro* produkce embryí skotu, který zahrnuje tři hlavní kroky: *in vitro* maturaci (IVM) oocytů, *in vitro* fertilizaci (IVF) a následnou *in vitro* kultivace (IVC) embryí do stádia blastocysty, ve kterém jsou přenášena recipientkám. Přesto metoda *in vitro* produkce embryí dosahuje malé účinnosti. Jadernou maturaci do MII stádia podstoupí cca. 90% nezralých oocytů, 80% oocytů podstoupí IVF a vyvine se do stádia 2 buněk. Pouze 30-40% z původních oocytů dosáhne stádia blastocysty, ve kterém mohou být přeneseny do recipentky nebo zamrazeny pro další použití (Lonergan et al., 2006). Kvalita oocytu je hlavní faktor, který ovlivňuje počet získaných blastocyst, zatímco kultivační podmínky po fertilizaci jsou rozhodující pro jejich kvalitu (Rizos et al., 2002).

Nejdůležitější událostí během raného preimplantačního vývoje je embryonální genomová aktivace (EGA), kdy dochází k přechodu z využití transkriptů pocházejících z maternálního genomu oocytu na transkripty přepisované z embryonálního genomu. Pokud k této události nedojde, embryo není schopno pokračovat v dalším vývoji (Kanka, 2003). Do embryonálního vývoje skotu může zasahovat mnoho faktorů, které vedou k zastavení embryonálního dělení, tzv. embryonálnímu bloku, který se u skotu vyskytuje během čtvrtého buněčného cyklu nebo mezi čtvrtým a pátým buněčným cyklem (Memili a First, 2000; Meirelles et al., 2004). Příčinou embryonálního vývojového bloku může být neschopnost aktivovat transkripci, neschopnost překonat chromatinovou represi nebo reakce na poruchy způsobené prostředím (Betts a King, 2001). Lonergan et al. (2003) se změřil na studium transkriptů měnících se během preimplantačního vývoje, které jsou silně ovlivněny kultivačním prostředím.

Odhaduje se, že pro úspěšný preimplantační a raný fetální vývoj je důležitá exprese asi 10 000 genů (Niemann a Wrenzycki, 2000), ale např. u myši, nejlépe prozkoumaného modelového organismu bylo identifikováno asi 15 700 genů exprimovaných v preimplantačním vývoji (Stanton et al., 2003). Identifikace a charakterizace genů, které jsou rozdílně regulované v oocytech a embryích je podstatná k porozumění kritických událostí vyskytujících se během raného vývoje. Zvířecí modely mohou v tomto směru poskytnout důležité informace pro studium účinků kultivace na vývoj savčích embryí, včetně lidských.

## 1.1. ČASNÝ EMBRYONÁLNÍ VÝVOJ

Preimplantační vývoj zahrnuje období od oplození po implantaci do děložní sliznice a je charakterizován množstvím významných vývojových událostí (Edwards, 2003). Mezi tyto události patří načasování prvního dělení, aktivace embryonálního genomu, kompaktace a formace blastocysty. Počátek preimplantačního vývoje je kontrolován mRNA maternálního původu (Lequarre et al., 2003).

### 1.1.1. TRANSKRIPCE A TRANSLACE BĚHEM MEIÓZY OOCYTU

Raný preimplantační vývoj je z velké míry podmíněn událostmi před oplozením a vznikem zygoty, zejména růstem a maturací oocytu, během které dochází k akumulaci maternální mRNA a proteinů. Rostoucí oocyt, zastavený v dipolotením stádiu I. meiotické profáze, je transkripčně a translačně aktivní (Schultz a Heyner, 1992; Wassarman a Kinloch, 1992). Tomek et al. (2002) zkoumali vztah mezi transkripcí, translací a polyadenylací mRNA během *in vitro* maturace bovinních oocytů. Jejich studie ukázala, že během meiotické maturace od stádia profáze (stádium zárodečného váčku, GV stádium) je stimulována celková proteinová syntéza na třínásobek po 6-10 h, nárůst se vyskytuje paralelně k počátku GVBD (rozpadu membrány zárodečného váčku) a pokračuje až do 14 h maturace, kdy většina oocytů již dokončila GVBD. V metafázi II inkorporace [$^{35}$S]metioninu do proteinu vykazuje základní hladinu translace jako ve stádiu GV. Měření inkorporovaného [$^{3}$H]uridinu do RNA v průběhu IVM ukazuje, že se de novo transkripce silně snižuje s nástupem GVBD (do GVBD je oocyt transkripčně aktivní) a v MII oocytech je transkripce na hranici detekce. Inkorporace [$^{3}$H]adenosinu, která odráží polyadenylační události, vzrůstá v časovém intervalu 6-10 h a v dalším průběhu maturace mírně klesá. Počátek GVBD je doprovázen nejen zvýšenou translací, ale také cytoplazmatickou polyadenylací mRNA, což potvrzuje hybridizace [$^{3}$H]poly(U) k cytoplazmatické frakci IVM oocytů (detekuje pouze poly(A) konec delší než 20 adenosinových residuí, typický pro mRNA). Tyto výsledky naznačují buď akumulaci poly(A) mRNA nebo prodlužování poly(A) konců během IVM, protože ve stejných časových intervalech byla transkripce silně snížena. V oocytu, který je zastavený v metafázi II. meiotického dělení, je zastavena transkripce a redukována translace mRNA (Bachvarova, 1992).

Po oplození oocytu dochází k dokončení meiózy a vzniku 1-buněčného embrya, které obsahuje haploidní samčí pronukleus ze spermie a haploidní samičí pronukleus z oocytu. Před vstupem do první mitózy podstoupí každý pronukleus DNA replikaci a následně

vzniká 2-buněčné embryo, které obsahuje dvě diploidní jádra, každé se sadou paternálních a maternálních chromozomů (Laurincik et al., 1998).

### 1.1.2. NAČASOVÁNÍ PRVNÍHO DĚLENÍ

Čas prvního dělení embrya má významný dlouhodobý účinek na další vývoj embrya do stádia blastocysty. Embrya dělící se ze stádia zygoty do 2-buněčného stádia dříve mají větší vývojový potenciál pro dosažení stádia blastocysty než později se dělící embrya. Tento fakt lze využít k neinvazivnímu hodnocení životaschopnosti embrya před transferem nebo zamrazením a nabízí se jeho využití i v humánní asistované reprodukci před přenosem embryí (Lonergan et al., 1999). Studie, zaměřená na souvislost mezi vývojovou kompetencí bovinního embrya, stanovenou jako čas prvního dělení a expresí genů pro receptor a ligand insulinu podobného růstového faktoru 1 (*insulin-like growth factor-I, IGF-I*), hypoxanthine fosforibosil transferázu (*HPRT*) a glukóza-6-fosfát dehydrogenázu (*G6PD*) prokázala vztah mezi časem prvního dělení a množstvím mRNA *G6PD* a *HPRT*. Zygoty, které se dělily 27 až 30 h po inseminaci, měly více mRNA pro tyto geny, než zygoty, dělící se 33 h po oplození. *IGF-I* receptor byl detekován ve všech časech dělení zygot, naproti tomu *IGF-I* ligand byl nalezen ve všech 2-buněčných embryích dělících se mezi 27 a 30 h po inseminaci, jen v části embryí dělících se mezi 33 a 36 h po inseminaci a žádný v embryích dělících se po 36 h. To ukazuje na rozdíly v genomové expresi, které odrážejí rozdíly vývojové kompetence raného a pozdního dělení zygot (Lonergan et al., 2000).

Vývojová kompetence a čas prvního dělení embrya je také ve vztahu k polyadenylačnímu stavu některých maternálních transkriptů. Brevini et al. (2002) analyzovala změny v polyadenylaci mRNA u bovinních oocytů a embryí od znovuzahájení meiózy až do prvního dělení embrya. Zejména byla zkoumána délka poly(A) konce transkriptů izolovaných z embryí, která dosáhla 2-buněčného stádia v rozdílných časových intervalech (27, 30, 36 a 42 h po inseminaci) a embryí, která se nedělila do 42 h po inseminaci. Polyadenylační stav byl zkoumán u genů, které charakterizují diferenciaci (*Oct-4*), kompaktaci a kavitaci (*β-actin, plakophilin, connexin-32, connexin-43*), energetický metabolismus (*glukose transporter type1, puryvat dehydrogenace phosphatase*), zpracovnání RNA (*RNA poly(A) polymerase*) a stres (*heat shock protein 70*). Mezi oocyty a prvním dělením v 27 h byl poly(A) konec beze změn u transkriptů *β-actin* a *PDP*, postupně redukován u *Cx43*, *Oct-4* a *Plako*, postupně prodloužen u *Cx-32* a *TPA*, či redukován a následně prodloužen u *PAP*, *HSP-70* a *Glut-1*. Pokud byl interval mezi inseminací a prvním dělením delší než 27 h, bylo u některých genů pozorováno další prodloužení nebo zkrácení poly(A) konce. Tyto výsledky ukazují, že špatná vývojová kompetence je doprovázena abnormální úrovní polyadenylace specifických maternálních mRNA.

### 1.1.3. MOLEKULÁRNÍ ZÁKLADY AKTIVACE EMBRYONÁLNÍHO GENOMU

Během preimplantačního vývoje byly pozorovány dvě vlny de novo transkripce První vlnou je označována embryonální genomová aktivace (EGA) a druhou vlnou je mid-preimplantační genomová aktivace (MGA), která uvádí dynamické morfologické a funkční změny ze stádia moruly do stádia blastocysty (Hamatani et al., 2004).

**Embryonální genomová aktivace (EGA)**

Proteiny a mRNA, které jsou naakumulovány během růstu a maturace oocytu umožní vývoj embrya ranými stádii preimplantačního vývoje až do chvíle, kdy si embryo začne produkovat vlastní mRNA a proteiny. Tento moment, kdy embryo začíná svojí vlastní transkripci, se nazývá embryonální genomová aktivace (EGA) a jeho načasování je druhově specifické (Kanka, 2003).

EGA se skládá nejméně z několika fází, které umožní další pokračování vývoje embrya. Patří sem degradace maternálně zděděných proteinů a mRNA včetně oocyt-specifických transkriptů, které nejsou v dalším vývoji exprimovány. Dalším úkolem EGA je nahradit maternální transkripty takzvaných „housekeeping" genů (např. aktin), které jsou společné oocytu i embryu, transkripty embryonálními. Třetím úkolem je celkové reprogramování a změna vzorce genové exprese, které vede k syntéze nových transkriptů specifických pro embryo (Schultz, 2002). Degradace maternální mRNA je postupný proces, který graduje kolem období EGA. Na myším modelu bylo zjištěno, že ve stádiu 2 buněk zůstává pouze 10-20 % počátečního množství maternální mRNA (Yokoi et al., 1993).

U savců dochází k zahájení EGA postupně ve dvou fázích. Majoritní aktivaci genů (major genome activation) předchází počáteční minoritní aktivace genů (minor genome activation). K majoritní aktivaci embryonálního genomu dochází u králíka ve stádiu 8 - 16 buněk, u člověka ve stádiu 4 - 8 buněk, u prasete ve stádiu 4 buněk a u myši k EGA dochází ve stádiu 2 buněk (Telford et al., 1990).

Na myši byla prokázána minoritní transkripční aktivita (EGA) inkorporací BrUTP [5-bromouridine 5'-trifosfátu] již v 1-buněčném embryu, 1-2 hodiny po počátku S fáze. Transkripční aktivita byla v samčím pronukleu 4x až 5x vyšší než v samičím (Aoki et al., 1997). První sytéza mRNA a proteinů v bovinních embryích byla zjištěna inkorporací [$^{3}$H]uridinu a [$^{35}$S]metioninu v 1-buněčném a 2-buněčném stádiu (Memili a First, 1999). Značení pomocí [$^{35}$S]UTP prokázalo stálou úroveň inkorporace od 2-buněčného stádia do 8-buněčného stádia, kde docházelo k opětovnému nárůstů inkorporace, což naznačuje majoritní aktivaci genů (Memili et al., 1998). S pomocí Affymetrix GeneChip Bovine Genome Array byl u *in vivo* bovinních embryí nalezen největší rozdíl v raném stádiu vývoje mezi oocyty a 4-buněčným stádiem a v pozdějším stádiu vývoje mezi 8-buněčným stádiem a blastocystou. To odráží přechod z maternálního genomu na embryonální. Mikročipy ukazují, že k minoritní genomové aktivaci dochází mezi stádii 2 – 4 buněk, později mezi stádii 4 a 8 buněk bylo nalezeno největší množství rozdílně exprimovaných genů, což demonstruje spuštění embryonální genomové aktivace (Kues et al., 2008). Kultivace bovinních oocytů a preimplantačních embryí v médiu obsahujícím [$^{35}$S]metionin ukazuje pokles syntézy proteinů mezi stádiem zygoty a 8-buněčným stádiem. Ke změně syntézy proteinů dochází na úrovni 8 – 16 buněk stádia vývoje. Syntéza proteinů postupně stoupá až do stádia blastocysty, kde dosahuje dvacetinásobku oproti 8-buněčnému stádiu (Frei et al., 1989).

Protože mRNA je přepisována pomocí RNA polymerázy II, využívá se často pro studium transkripce v preimplantačním vývoji specifický inhibitor této RNA polymerázy

α-amanitin. U bovinních embryí inhibice RNA polymerázy II během prvních 4 buněčných cyklů ovlivňuje průběh embryonálního vývoje od 16-buněčného stádia. To lze využít k zjištění významu raných transkriptů, které jsou nezbytné pro další vývoj embrya (Memili a First, 1998). Pokud je α-amanitin přidán do kultivačního média bovinních embryí ve stádiu 4 buněk a embrya se v jeho přítomnosti kultivují do stádia moruly, dochází ke kompletní inhibici dalšího vývoje (Liu a Foote, 1997).

Důležitou roli při reprogramaci genové exprese během EGA hraje také remodelace chromatinu (Kanka, 2003). Histon acetyltransferázy (HAT) katalyzují acetylaci konců nukleozomů a tím uvolňují chromatin, což napomáhá přístupu transkripčních faktorů a umožňuje tak transkripci genů (Marmorstein, 2001). V somatických buňkách je aktivní genová exprese spojena s acetylací histonů H3 a H4, stejné modifikace se ale vyskytují i v transkripčně neaktivních oocytech (Fulka et al., 2008). Práce Sarmento et al. (2004) naznačuje, že cytoplasma oocytů a raného embrya obsahuje histon deacetylasy a pravděpodobně i histon arginin demetylázy, jejichž aktivita se může podílet na reprogramaci genomu během EGA.

**Mid-preimplantační genomová aktivace (MGA)**

První morfogenetickou událostí během preimplantačního vývoje je kompaktace (Watson a Barcroft, 2001; Stanton et al., 2003). Kompaktace je stádium ve kterém se do té doby volné sférické blastomery spojují k sobě a vytváří pevně organizovanou buněčnou masu, morulu. U myši ke kompaktaci embryí dochází již v 8-buněčném stádiu, kdy začíná adheze buněk zprostředkovaná E-cadheriny. Buňky se navzájem kompaktují (stlačují), zmenšují mezibuněčné prostory a dochází k formaci mezibuněčných spojů: tight junctions, které jsou podstatné pro zadržení blastocelové tekutiny a udržují epiteliální buněčnou polaritu a gap junctions, které zajišťují mezibuněčnou komunikaci (Becker et al., 1992; Watson et al., 1999). U *in vivo* produkovaných bovinních embryí začíná kompaktace kolem 32-buněčného stádia (od pátého do sedmého buněčného cyklu)(Van Soom et al., 1997).

Maternální zásoba E-cadherinu umožňuje kompaktaci i u myších embryí s knock-outovaným *E-cadherinem* (Watson et al., 1999), ale pro vývoj do blastocysty a hatching je de novo syntéza E-cadherinu nezbytná. Přítomnost mRNA kódující *connexin 43* (*Cx43*), jeden z proteinů tvořících gap junctions, byla stanovena v bovinních embryích *in vivo* a *in vitro*. Transkript *Cx43* byl nalezen u *in vivo* embryí od stádia oocytů až do stádia blastocysty, zatímco v podmínkách *in vitro* byl identifikován pouze do stádia moruly, ale nebyl detekován v blastocystách a hatched blastocystách, což naznačuje nedokonalost *in vitro* kultivačních podmínek (Wrenzycki et al., 1996). Exprese *Cx43* se mění v přítomnosti séra v médiu, kdy transkripce mizí ve stádiu 8 – 16 buněk a znovu se objevuje ve stádiu hatched blastocysty (Wrenzycki et al., 1999). Bloor et al. (2004) srovnávali expresi konexinů během preimplantačního vývoje u člověka a hlodavců a potvrdili přítomnost mnoha konexinových isoforem na obou modelech. To naznačuje podobnou regulaci konexinů a jejich význam pro preimplantační vývoj savčího embrya.

Blastulace začíná u *in vivo* bovinních embryí po dosažení stádia 64 buněk v sedmém buněčném cyklu, hatching začíná obvykle během osmého buněčného cyklu. V podmínkách

*in vitro* dochází k tvorbě blastocysty dříve, již během šestého buněčného dělení a to mezi 32 a 64-buněčnými stádii (Van Soom et al., 1997).

Kavitace embrya a vytvoření blastocoelu je zprostředkováno transportem kapaliny přes vnější blastomery trofoektodermu (TE) a vyžaduje koordinaci exprese několika genových produktů zahrnující Na/K-ATPase podjednotky, aquaporiny (AQP) a podjednotky tight junction (např. ZO-1). (Watson et al., 1999). Dochází k formaci dvou embryonálních buněčných linií, zahrnujících vnější epiteliální vrstvu trofoektodermu (TE), který dá vzniknout extraembryonálním tkáním; a vnitřní inner cell mass (ICM), která je složena z pluripotentních buněk a dává vznik vlastnímu embryu (Watson et al., 1999). Wrenzycki et al. (2003) zkoumala na bovinním modelu genovou expresi v tomto období a potvrdila mimo jiné expresi transkriptů pro Na/K-ATPasy, E-cadherine a ZO-1 protein v expandovaných blastocystách. Formace blastocysty je nezbytná pro implantaci a udržení březosti a při *in vitro* kultivaci je měřítkem kvality embrya před přenosem (Watson et al., 1999; Watson et al., 2004).

### 1.1.4. STUDIUM EXPRESE A FUNKCE GENŮ V PRŮBĚHU VÝVOJE

**Studium exprese genů pomocí metody supresivní subtraktivní hybridizace (SSH)**

Technika supresivní subtraktivní hybridizace může být použita k charakterizaci transkriptomu a pozorování odchylek v mRNA expresi mezi dvěma populacemi. Vhodnost SSH pro obohacení cDNA knihoven o velmi vzácné transkripty exprimované během preimplantačního embryonálního vývoje byla ověřena studií Bui et al. (2005). V této studii byly připraveny tři cDNA knihovny transkriptů exprimovaných během embryonální genomové aktivace u skotu a králíka a další knihovna, která byla zaměřena na studium diferenciace ve stádiu blastocysty u králíka. K cDNA populaci, která měla být obohacena o vzácné transkripty (tester) byly přidány v malém množství exogenní transkripty původem z *Arabidopsis thaliana*. Další exogenní transkripty byly ve velkém množství přidány k oběma populacím cDNA (testeru i driveru) a měly tak reprezentovat běžné transkripty společné oběma populacím. Bylo prokázáno, že velmi vzácné transkripty byly ve výsledných SSH knihovnách obohaceny, i když nedošlo k úplné normalizaci jejich množství vůči běžně zastoupeným transkriptům. To potvrzuje vhodnost SSH jako metody pro detekci dosud neznámých transkriptů ze vzorků s malým obsahem RNA, jako jsou oocyty a preimplantační embrya savců.

Fair et al. (2004) pomocí metody SSH identifikovali mRNA transkripty spojené s embryonální vývojovou kompetencí u skotu. Identifikace byla provedena srovnáním dvou populací 2-buněčných embryí: embrya s raným dělením (dříve než 24 h po inseminaci) a pozdním dělením (později než 33 h po inseminaci). Pro další analýzu pomocí metody kvantitativní RT-PCR byly vybrány 3 geny (*histone H3, cyclin B1* a *GDF-9B*), které hrají klíčovou roli v preimpalntačním vývoji a pomocí SSH byly identifikovány jako zdánlivě up-regulované v raně se dělících zygotách. Embrya pro analýzu byla brána z kultivace v 3 h intervalech (25, 28, 32 a 36 h nebo starší 36 h po inseminaci). Analýza kvantitativní RT-PCR potvrdila signifikantně významnou vyšší úroveň exprese *histonu H3* v dříve se dělícím embryu, tato exprese ale s prodlouženým časem dělení klesala.

Další studie, která se zabývala identifikací genů souvisejících s vývojovou kompetencí a časem prvního dělení embrya podrobně mapovala expresi 16 transkriptů v raně a pozdně se dělících 2-buněčných embryích. Studované transkripty byly vybrány na základě jejich předešlé studie pomocí metody SSH mezi ranými a pozdně se dělícími embryi. Pomocí metody kvantitativní RT-PCR byly identifikovány 3 geny (*YEAF, IDH, H2A*) statisticky významně (P<0,05) více exprimované v raně se dělícím embryu, jejichž exprese může být spojena s vyšší vývojovou kompetencí. Další tři geny (*TCP1, cathepsin B, RAD50*) vykazovaly expresi zvýšenou o více než 30 % oproti pozdně se dělícím embryím, ale tento výsledek byl statisticky významný s P<0,1 (Dode et al., 2006).

Srovnání hatchovaných bovinních blastocyst (9-10 den *in vitro*, tester) s intaktními blastocystami (7-8 den *in vitro*, driver) pomocí SSH identifikovalo 31 transkriptů více exprimovaných v hatchovaných blastocystách. Mezi identifikovanými geny byly tři (*26S proteasomal ATPase, PSMC3; casein kinase 2α subunit, CK2; phosphoglycerate kinase, PGK*) s předpokládanou funkcí během rané embryogeneze. Tyto geny byly dále charakterizovány pomocí kvantitativní RT-PCR. U všech tří genů byl potvrzen nárůst exprese v hatchovaných blastocystách. Tato studie může poskytnout informace o genové expresi během preimplantační embryogeneze (Mohan et al., 2002).

SSH byla použita také k porovnání expresních rozdílů mezi *in vivo* a *in vitro* získanými blastocystami skotu. Pro charakterizaci pomocí kvantitativní RT-PCR byly vybrány dva geny z knihovny obohacené o transkripty více zastoupené *in vivo* (*galectin-1* a *fibronectin*), a jeden z knihovny transkriptů více zastoupených *in vitro* (*filamin A*). Metoda kvantitativní RT-PCR potvrdila signifikantní nárůst exprese transkriptů pro *galectin-1* a *fibronectin* v *in vivo* blastocystách v porovnání s *in vitro* kultivovanými blastocystami. U genu *filamin A* nebyl nalezen signifikantní rozdíl v expresi mezi *in vivo* a *in vitro* kultivovanými blastocystami. Charakteristika odlišností v genové expresi mezi *in vivo* a *in vitro* získanými embryi může zlepšit porozumění ranému embryonálnímu vývoji a napomoci zdokonalení *in vitro* kultivačního systému (Mohan et al., 2004).

**Studium exprese genů pomocí mikročipové technologie**

Mikročipové technologie umožňují stanovit transkripční profil mnoha (tisíce) exprimovaných genů najednou v určitých experimentálních stádiích či kultivačních podmínkách, nebo pomocí mezidruhové homologie nalézt evolučně konzervované geny.

Pro identifikaci evolučně konzervovaných genů exprimovaných v oocytech byl použit multi-druhový cDNA mikročip, který obsahuje transkripty ze tří specifických cDNA knihoven oocytů skotu, myši a *Xenopus laevis*, připravených pomocí SSH (Vallée et al., 2006). Z každé ze tří knihoven, připravených subtrakcí oocytů a směsi somatických tkání, bylo náhodně vybráno 1152 klonů a ty byly natištěny na mikročip. K takto připravenému mikročipu pak byly postupně hybridizovány všechny cDNA klony ze všech tří druhově specifických knihoven. Tímto postupem bylo identifikováno 1541 transkriptů, které byly exprimovány v oocytech všech tří druhů. Porovnáním těchto transkriptů se seznamem transkriptů preferenčně exprimovaných v oocytech byl získán seznam 268 evolučně konzervovaných

transkriptů preferenčně exprimovaných v oocytech. Mezi takto nalezenými transkripty bylo již o několika známo, že jsou přítomny v oocytech, například *zona pellucida glycoprotein 2 (Zp2), growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (Bmp15)* a další. Pro ověření dat získaných mezidruhovou hybridizací byly vybrány tři oocyt specifické transkripty, u kterých nebyla dosud známá exprese u dvou ze tří zkoumaných druhů: *small fragment nuclease* (*SMNF*), nalezená v bovinních oocytech, *spindlin* (*Spin*), nalezený v myších oocytech a *protein arginin methyltransferase 1* (*PRMT1*), identifikovaná v ocytech *Xenopus laevis*. Pro tyto transkripty byly navrženy druhově specifické primery a pomocí RT-PCR byla identifikována jejich exprese v oocytech druhů, kde předtím nebyly prokázány, např. *spindlin* byl takto detekován v oocytech skotu a *X. laevis*. Tyto výsledky potvrzují sílu mezidruhových mikročipových studií pro identifikaci evolučně konzervovaných genů.

Další mezidruhová mikročipová studie, ve které byly bovinní oocyty a embrya ve stádiu blastocysty hybridizovány na humánní mikročip poskytla informace o evolučně konzervovaných genech během vývoje skotu a člověka. V oocytech byly nalezeny geny týkající se transkripční a translační kontroly (*ELAVL4, TACC3*), zatímco v blastocystách to byly transkripty týkající se buněčného transportu (*SLC2A14, SLC1A3*), proteazomu (*PSMA1, PSMB3*), buněčného cyklu (*BUB3, CCNE1, GSPT1*) a proteinových modifikací (*TNK1, UB3A*). Geny týkající se chromatinové remodelace byly nalezené v obou stádiích, v oocytech (*NASP, SMARCA2*) i v blastocystách (*H2AFY, HADC7A*) (Adjaye et al., 2007).

Pomocí Affymetrix GeneChip Bovine Genome Array s 23 000 transkripty byla analyzována genová exprese mezi oocyty v MII fázi, embryi v 8-buněčném stádiu a embryi v 8-buněčném stádiu kultivovanými s α-amanitinem. V 8-buněčném stádiu byly více exprimovány geny, které regulují transkripci, chromatinovou strukturu, buněčnou adhezi a signální transdukce, ve srovnání s 8-buněčnými embryi ošetřenými α-amanitinem a oocyty v MII fázi. V oocytech v MII fázi byly více exprimovány geny kontrolující DNA metylaci a metabolismus. Pomocí kvantitativní RT-PCR byla potvrzena exprese několika genů (*NFYA transkripční faktror T- α, H2AFZ, SWI/SNF-related SMARCAL1*) se vzrůstající expresí v 8-buněčném stádiu. Z oocyt specifických transkriptů byla potvrzena exprese *IGF2R insulin-like growth factor*, *DNMT1 DNA (cytosin5) methyltransferase1* a *STAT3* (Misirlioglu et al., 2006).

Bovinní oocyty v MII fázi a všechna stádia *in vivo* preimplantačního vývoje skotu až do stádia blastocysty byly studovány také pomocí Affymetrix GeneChip Bovine Genome Array. Získaná data ukazují, že bovinní oocyty a embrya transkribují výrazně vyšší počet genů než somatické buňky. Autoři nalezli přibližně 350 genů, které byly transkribovány před 8-buněčným stadiem, tedy před hlavní genomovou aktivací. Pomocí kvantitativní RT-PCR se pokusili potvrdit tuto transkripci u 6 vybraných genů. U pěti z nich výsledky kvantitativní RT-PCR kopírují výsledky získané na mikročipu. Autoři však nepoužívají kontrolu s α-amanitinem, takže je obtížné rozhodnout, zdali nalezená mRNA je skutečně syntetizována z embryonálního genomu. Autoři rovněž detekovali expresi genů, charakteristickou pro 2-buněčné, 4-buněčné, 8-buněčné stadium, morulu a blastocystu, což naznačuje existenci

dynamických změn embryonálního transkriptomu a změn ve skupinách krátkodobě se exprimujících genů. Mikročipová analýza odhalila expresi *ZP2, ZP3, ZP4, GDF9, BMP15, cadherine (CDH3), Oct4 (POU5F1), CLDN, ZO3* a *TP53* v oocytech. Dále data ukazují, že minoritní genomová aktivace probíhá mezi 2-buněčným a 4-buněčným stádiem s up-regulací těchto genů: *SARS, IL18, CRABP1, ACO2, TXN2, SLC38A2, SLC25A3*. V 8-buněčném stádiu byly up-regulovány zejména translační iniciační faktory (*EIF2, EIF 3, EIF 4* a *EIF 5*). Největší rozdíly byly nalezeny mezi oocyty a 4-buněčným stádiem, v pozdějším vývoji mezi 8-buněčným stádiem a stádiem blastocysty. Ovšem největší množství rozdílně regulovaných genů bylo mezi 4-buněčným stádiem a 8-buněčným stádiem, což demonstruje hlavní počátek embryonální genomové aktivace v 8-buněčném stádiu. Maternální expresní profil genů (*DNMT1, ZP2, GDF9*) a embryonální expresní profil genů (*GLUT8, DNMT3A, VDAC*) byl potvrzen pomocí metody kvantitativní RT-PCR. (Kues et al., 2008).

Analýza exprese *in vivo* a *in vitro* maturovaných oocytů pomocí Affymetrix GeneChip Bovine Genome Array identifikovala pomocí klastrovací analýzy několik biologických procesů ovlivněných *in vitro* maturací. Mezi tyto procesy patřily zejména dráhy zahrnující metabolismus, produkci energie, buněčnou organizaci a biogenezi, buněčný růst a homeostázi (Katz-Jaffe et al., 2009).

**Studium funkce genů pomocí metody RNA interference (RNAi)**

Jako RNA interference (RNAi) se označuje potlačení exprese specifického genu pomocí vnesení odpovídající dsRNA. Tento jev byl poprvé v živočišné říši popsán u nematoda *C. elegans* (Fire et al., 1998). Postupně se podařilo prokázat efektivitu tohoto přístupu u dalších mnohobuněčných organismů, například *D. melanogaster*, ale možnost využití tohoto jevu u savců byla zpochybňována. Vnesení dsRNA do savčích buněk obvykle aktivuje interferonovou odpověď a vede ke globálnímu potlačení translace a destrukci buňky apoptózou (Lee a Esteban, 1994). Protože u savčích oocytů a preimplantačních embryí k interferonové odpovědi nedochází, byl to právě tento systém, na kterém byla efektivita RNAi u savců demonstrována. V roce 2000 Wianny a Zernicka-Goetz potlačily u myšího oocytu a embrya expresi maternálního genu *c-mos*, zygoticky exprimovaného *E-cadherine* a *GFP* transgenu. Svoboda et al. (2000) ve stejném roce potlačil pomocí mikroinjekce dsRNA transkripty pro *c-mos* a *Plat* v myších oocytech. Postupně bylo zjištěno, že vlastním efektorem RNAi není dsRNA, ale molekuly siRNA (small interfering RNA) a miRNA (micro RNA). Molekuly siRNA (small interfering RNA) o délce 21-23 nukleotidů, které jsou schopny potlačit expresi genu na základě komplementární sekvence k cílové mRNA, jsou produkovány z dsRNA exogenního původu (Sandy et al., 2005). MicroRNA (miRNA) jsou malé nekódující RNA endogenního původu, s délkou 21 – 25 nukleotidů (He a Hannon, 2004), transkribované RNA polymerázou II (Cai et al., 2004). Molekuly miRNA mohou pomocí vazby k 3´ nepřekládané oblasti (3´ UTR) potlačovat genovou expresi buď na základě úplné komplementarity k cílové mRNA, která spustí degradaci mRNA (Zeng et al., 2003), nebo na základě částečné sekvenční komplementarity, která vede k následné inhibici translace. Během vývoje preimplantačního embrya se miRNA účastní degradace maternální mRNA. Vazba microRNA a proteinů na

3´UTR oblast cílových mRNA pomocí sekvenční komplementarity vede k potlačení translace těchto mRNA a urychlení jejich rozkladu. Na cílové mRNA navodí deadenylaci a tím zvýší její náchylnost k degradaci nukleázami. Degradací se odstraní genové produkty, které by mohly později překážet v dalším vývoji embrya (Schier, 2007). Kromě této přirozené role ve vývoji embrya se dnes efektu RNAi používá jako cenného nástroje pro studium genové funkce. Vnesení dsRNA do oocytu nebo embrya pomocí mikroinjikace nebo transfekce tak umožňuje sledování změn exprese specifických genů přímo na jednotlivých oocytech nebo embryích. Paradis et al. (2005) využil RNAi k potlačení exprese *cyklinu B1* během zrání bovinních oocytů, Nganvongpanit et al. (2006b) pomocí této techniky umlčel v bovinních oocytech a embryích geny *C-mos*, respektive *Oct4*. Potlačení exprese *E-cadherine* (Nganvongpanit et al., 2006a) během preimplantačního vývoje bovinního embrya významně snížílo počet získaných blastocyst a tak potvrdilo význam *E-cadherine* pro formaci blastocoelu. V naší laboratoři jsme použili injikaci dsRNA pro umlčení genu *CENPF* a objasnění jeho role během preimplantačního vývoje.

## 1.2. POUŽITÉ METODY

### 1.2.1. SUPRESIVNÍ SUBTRAKTIVNÍ HYBRIDIZACE (SSH)

Supresivní subtraktivní hybridizace (suppression subtractive hybridization, SSH) je metoda, která umožňuje porovnat dvě populace DNA a zkonstruovat DNA knihovnu obohacenou o sekvence přítomné pouze v jedné z populací. S využitím reverzní transkripce lze SSH použít ke zkonstruování cDNA knihovny obohacené o rozdílně exprimované geny mezi dvěma vzorky mRNA. Oba mRNA vzorky jsou reverzně transkribovány a následně amplifikovány. Vzorek cDNA, který chceme obohatit o transkripty nepřítomné v druhé referenční populaci, je nazýván tester a referenční cDNA driver. Tester je rozdělen na dvě části a každá část je ligována s jiným adaptorem. Tester a driver jsou spolu hybridizovány ve dvou po sobě jdoucích hybridizacích. První hybridizace je provedena odděleně pro obě poloviny testeru ligované s různými adaptory a to tak, že je driver v nadbytku k testeru. cDNA je denaturována, následuje annealing a vytvoření hybridů mezi testerem a driverem. Při druhé hybridizaci jsou produkty obou primárních hybridizací smíchány dohromady. V této fázi dojde k hybridizaci sekvencí, které nebyly přítomné v driver cDNA. Následuje exponenciální amplifikace cDNA molekul testeru v PCR reakci navržené tak, aby amplifikovala pouze sekvence, které obsahují oba adaptory (Diatchenko et al., 1996). Po amplifikaci jsou produkty klonovány do PCR 2.1 plazmidu a transfekovány do chemicky kompetentních *Escherichia coli*, izolovány, osekvenovány a porovnány v BLAST programu s NCBI (National Center for Biotechnology Information) GenBank databází (Fair et al., 2004).

Metodu SSH lze použít přímo ke zjištění genů exprimovaných specificky v jednom vzorku oproti jinému, nebo mohou vytvořené PCR produkty sloužit k naspotování na mikročip (Sirard et al., 2005).

### 1.2.2. VYUŽITÍ MIKROČIPŮ PRO DETEKCI EXPRIMOVANÝCH GENŮ

Mikročipy se používají k monitorování exprese mnoha genů najednou na základě komplementarity sekvencí nukleových kyselin. Úseky DNA (sondy) komplementární k detekované sekvenci (cíl, target), jsou ručně nebo pomocí speciálních robotů natištěny na nosič (mikročip), tvořený mikroskopickým sklíčkem se speciálním povrchem (Schena et al., 1995) nebo nylonovou membránou. K takto připravenému mikročipu, který obsahuje stovky až tisíce sond, je pak hybridizován fluorescenčně nebo radioaktivně značený vzorek a následně je kvantifikována síla signálu pro každou jednotlivou sondu na mikročipu. Podle délky sekvence sond rozeznáváme cDNA mikročipy, kde je na povrchu čipu naspotována celá cDNA sekvence komplementární k detekované RNA, a oligonukleotidové mikročipy, které využívají jen kratších sond (obvykle 25-70 bp) navržených tak, aby byly specifické pouze pro cílovou sekvenci. Zástupcem komerčně dostupných oligonukleotidových mikročipů je např. Affymetrix, který využívá 25 bp dlouhé oligonukleotidy syntetizované in situ (Schinke-Braun a Couget, 2007) či Agilent, který produkuje mikročipy s roboticky natištěnými 60 bp oligonukleotidy. Ve své práci jsem využívala cDNA mikročip specificky navržený pro studium preimplantačního vývoje u skotu (Sirard et al., 2005), založený na PCR produktech z cDNA knihoven připravených sérií subtraktivních hybridizací od oocytů po blastocystu.

Na každý mikročip je možné hybridizovat buď jeden vzorek (jednokanálové mikročipy), nebo vzorky dva, přičemž každý je značen jiným fluorochromem (např. Cy3 a Cy5, dvoukanálové mikročipy). Jako jednokanálové jsou typicky používány mikročipy Affymetrix, analýza rozdílů v genové expresi pak spočívá v porovnání intenzity signálu mezi jednotlivými mikročipy. Mikročipy firmy Agilent a většina nekomerčně tištěných cDNA mikročipů je většinou používána v dvoukanálovém uspořádání. To přináší výhodu v možnosti srovnat rozdíly v genové expresi mezi dvěma vzorky přímo v rámci jednoho mikročipu, tedy s menší variabilitou a s větším statistickým významem než je srovnání mezi mikročipy. Nevýhodou je složitější experimentální design při potřebě srovnat více než dva vzorky, nebo v případě, že je potřeba do experimentu zahrnout nové vzorky dodatečně. Důležitou součástí každého mikročipového experimentu je příprava vzorku. Ta v případě expresních mikročipů zahrnuje izolaci mRNA ze vzorku a její označení, případně amplifikaci, pokud je izolované množství mRNA malé. Značení vzorků může být přímé či nepřímé. U přímého značení se inkorporují fluorescenčně značené nukleotidy (např. Cy-UTP) přímo do reakce reverzní transkripce (cDNA) nebo *in vitro* transkripce (aRNA), u značení nepřímého do reakce vstupují nukleotidy modifikované přítomností reaktivní skupiny, ke které se fluorescenční značka naváže v samostatné reakci. Například 5-(3-aminoallyl)-dUTP (aa-dUTP) po inkorporaci umožňuje reakci modifikované cDNA nebo aRNA s NHS estery fluorescenčních barev Cy3 nebo Cy5 (t Hoen et al., 2003), u mikročipů Affymetrixu se využívá nepřímé značení biotinem a jeho vysoké afinity k streptavidinu.

Na jeden mikročip je obvykle hybridizováno cca. 2,5-10 μg vzorku. V mnoha případech je množství dostupného vzorku příliš malé pro přímé využití v mikročipové analýze a je nutné vzorek předem amplifikovat. Amplifikace vzorku může být lineární nebo exponenciální

(Nygaard a Hovig, 2006; Subkhankulova a Livesey, 2006) a může být opakována i několikrát po sobě. Zvláště v případě mikročipové analýzi savčích oocytů a embryí je vzhledem k malému množství celkové RNA v jednom oocytu (cca. 2.4 ng) či v embryu (cca. 5 ng v blastocystě) (Bilodeau-Goeseels a Schultz, 1997) nutná příprava směsných vzorků z více embryí a jedno či dvoukroková amplifikace. Základním principem mikročipů je hybridizace značených vzorků se sondami na povrchu mikročipu, přičemž u dvoukanálových mikročipů jsou oba vzorky aplikovány najednou ve stejném množství. Po hybridizaci a odmytí nehybridizovaných nebo nespecificky hybridizovaných sekvencí je nutné odečíst kvantitu signálu pomocí leaserového scanneru. Během skenování je důležité nastavení správného rozlišení, napětí fotonásobiče a intenzity laseru tak, aby byla přibližně stejná průměrná síla signálu u všech mikročipů a žádná z jednotlivých teček (spot) by neměla být saturována. V průběhu skenování dvoukanálových mikročipů by měl být poměr signálu pro oba kanály (Cy3, Cy5) kolem 1.

Analýza mikročipových dat zahrnuje analýzu obrazu, normalizaci a statistické zpracování dat. Analýza obrazu spočívá v převedení obrazové informace získané skenováním na číselnou intenzitu signálu pro každou sondu přítomnou na čipu a to ve všech v experimentu použitých kanálech. Účel normalizace spočívá v potlačení odchylek měření, které mohou vznikat z rozdílů mikročipové technologie jako jsou rozdíly mezi červenou a zelenou barvou vlivem různé účinnosti značení u dvoukanálových mikročipů a rozdíly v nastavení scaneru („*within slide*“ *normalization*, normalizace v rámci jednoho čipu), nebo rozdíly v celkové intenzitě signálu mezi jednotlivými mikročipy v experimentu („*between slide*“ *normalization*, normalizace mezi čipy) (Smyth a Speed, 2003). Zvolení vhodné statistické metody pro identifikaci rozdílně exprimovaných genů záleží na výsledných datech po normalizaci a na daném biologickém problému. Pro analýzu rozdílů exprese mezi vzorky z mikročipových dat lze použít mnoho komerčních i volně dostupných softwarů. Například v statistickém prostředí R lze využít některý z mnoha balíčků projektu Bioconductor, např. balíček limma, který používá lineární modely pro analýzu navrženého experimentu a hodnocení rozdílné exprese (Smyth, 2004). Sada TM4 http://www.tm4.org, je tvořena několika samostatnými programy, které svou funkcí pokrývají všechny základní kroky analýzy mikročipových expresních dat, tedy analýzu obrazu (Spotfinder), normalizaci (MIDAS) i analýzu rozdílů exprese mezi vzorky (MEV). Další často používanou metodou pro identifikaci rozdílně exprimovaných genů je Significance Analysis of Microarrays (SAM), založená na genově specifickém t-testu, který určuje skóre každého genu na základě změn v relativní genové expresi ke standardní odchylce opakovaných měření (Tusher et al., 2001). SAM je dostupný buď jako balíček pro statistické prostředí R nebo jako doplněk programu Microsoft Excel.

Vzhledem k velké variabilitě, která je dána v každé fázi přípravy mikročipového experimentu je potřeba výsledky mikročipových dat následně ověřit jinou nezávislou metodou, např. pomocí kvantitativní RT-PCR.

### 1.2.3. KVANTITATIVNÍ RT-PCR

Polymerázová řetězová reakce (PCR) je metoda založená na namnožení úseku DNA, který je vymezený specifickými primery (Mullis a Faloona, 1987; Saiki et al., 1988). Kvantitativní (real-time) PCR je založena na zaznamenávání každého reakčního cyklu v reálném čase za pomoci fluorescenčního značení (Higuchi et al., 1993; Bustin et al., 2005). Fluorescenční značení je buď pomocí DNA vázající barvy, nebo pomocí specifické DNA sekvence (sondy) s fluorescenčním reportérem. DNA vázající barvy, jako je SYBR Green, se vážou do dsDNA a způsobují zvýšení fluorescenčního signálu se vzrůstajícím množstvím PCR produktu (Wittwer et al., 1997). Specifické DNA sondy mohou být použité v multiplexu, kdy dochází k detekci několika genů v jedné reakci. Hydrolyzační sondy TaqMan využívají 5´-nukleázovou aktivitu DNA polymerázy k hydrolýze hybridizační sondy vazbou na cílový amplikon, to způsobí emisi fluorescence. Hybridizační sondy (FRET) jsou dvě fluorescenčně značené sondy, každá značená jiným fluorochromem, využívající rezonanční přenos energie k vybuzení fluorescence pouze v případě hybridizace obou sond k cílové sekvenci, což zajišťuje větší specificitu (Bustin, 2000). Hairpin sondy, jako je např. Scorpion, Molecular beacon jsou oligonukleotidy, které vytváří sekundární strukturu a přiblíží tak k sobě fluorochrom a molekulu zhášící fluorescenci. Po navázání takové sondy na detekovaný amplikon je zrušena původní sekundární struktura sondy a dochází k emisi fluorescence (Wong a Medrano, 2005). Pokud před PCR reakci zařadíme reverzní transkripci, můžeme pomocí kvantitativní PCR studovat i RNA vzorky. Mluvíme pak o kvantitativní RT-PCR (qRT-PCR). Prvním krokem qRT-PCR je reverzní transkripce, kdy RNA templát je reverzně transkribován do cDNA, která je exponenciálně amplifikována v PCR reakci za použití RNA- a DNA- dependentní DNA polymerázy a to buď jednokrokovou reakcí („2 enzymy/ 1 zkumavka“), kdy reverzní transkripce i amplifikace probíhá v jedné zkumavce nebo dvoukrokovou reakcí („2 enzymy/ 2 zkumavky“), při které probíhá reverzní transkripce a amplifikace zvlášť (Bustin, 2000).

Výsledkem qRT-PCR je kvantifikace fluorescence v každém cyklu reakce, která je úměrná množství produktu. Podle toho, zda ze získaných dat chceme zjistit přímo množství nukleové kyseliny v analyzovaném vzorku, nebo jen poměr tohoto množství k nějaké jiné nukleové kyselině ve stejném vzorku, mluvíme o absolutní nebo relativní kvantifikaci. Absolutní kvantifikace je založena na využití série ředění RNA o známé koncentraci k vytvoření standardní křivky. Vzhledem k vysokému dynamickému rozsahu, pro který je real-time RT-PCR detekce pomocí fluorescence lineární, je standardní křivku snadné zhotovit. Jako relativní standard slouží vzorek, z kterého se připraví série ředění pro vytvoření standardní křivky. Relativním standardem může být teoreticky jakákoliv nukleová kyselina, pokud známe její koncentraci a délku amplikonu, ideální je ale použít přímo sekvenci, která má být stanovena. Během real-time RT-PCR měření je Ct (treshold cycle) hodnota stanovovaného vzorku přímo porovnána s Ct hodnotou relativního standardu a je určeno, zda obsahuje více či méně mRNA, než dané ředění standardu (Bustin, 2000).

U relativní kvantifikace jsou výsledky pro jednotlivé geny normalizovány k relativní koncentraci referenčního vzorku. Jako referenční vzorek může být použita interní kontrola, u

které nepředpokládáme změnu v expresi během experimentu – housekeeping gen (Dheda et al., 2004), (např. *GAPDH, β-actin, β2-microglobulin*, a rRNA). Lze použít jeden vybraný gen nebo geometrický průměr několika housekeeping genů pro určení přesného profilu malých expresních rozdílů (Vandesompele et al., 2002). Další možností je relativní kvantifikace vzhledem k externímu standardu (Livak a Schmittgen, 2001). Jako externí standard lze použít mRNA (např. *luciferase*), která se přidává ve známé koncentraci ke vzorku před izolací RNA (Kanka et al., 2009). Pro statistickou analýzu expresních dat lze využít volně dostupné programy, jako je Q-Gene (Muller et al., 2002), pro relativní expresi statistický program REST (Pfaffl et al., 2002) nebo balíček pro analýzu qRT-PCR z projektu Bioconductor v statistickém prostředí R (Ritz a Spiess, 2008)

Kvantitativní RT-PCR je vysoce citlivá a výkonná technika ke kvantifikaci nukleových kyselin v biologickém vzorku (Huggett et al., 2005), která způsobila revoluci v měření genové exprese. Její citlivost umožňuje kvantifikaci nukleových kyselin z jednoho oocytu nebo embrya (Steuerwald et al., 1999) a je proto běžně využívána pro studium genové exprese v preimplantačním vývoji.

# 2. CÍLE PRÁCE

- **Pomocí supresivní subtraktivní hybridizace (SSH) vytvořit knihovny genů transkribovaných v bovinních embryích ve stádiu 4 a 8 buněk.**

- **Pomocí mikročipové technologie identifikovat rozdílně exprimované geny během minoritní a majoritní aktivace embryonálního genomu skotu v podmínkách** *in vivo* **a** *in vitro* **kultivace.**

- **Ověřit rozdíly v genové expresi zjištěné metodou SSH a mikročipů pomocí kvantitativní RT-PCR (qRT-PCR) a studovat expresi vybraných genů během celého preimplantačního vývoje.**

# 3. SOUHRN VÝSLEDKŮ A KOMENTÁŘE K VYBRANÝM PUBLIKACÍM

## 3.1. Kanka J, Kepkova K, Nemcova L.: Gene expression during minor genome activation in preimplantation bovine development. Theriogenology. 2009 Sep 1;72(4):572-83. Epub 2009 Jun 5

Hlavním cílem této studie bylo identifikovat mRNA transkripty nově syntetizované během bovinní minoritní genomové aktivace. Pomocí metody supresivní subtraktivní hybridizace (SSH) byla porovnána embrya ve stádiu 4 buněk (tester) vůči oocytům v MII fázi (driver). Produkty SSH byly klonovány do vektoru pCR2.1, transfekovány do kompetentních bakterií *E. coli* a z vyselektovaných klonů byly následně izolovány inzerty a na základě jejich délky (delší než 400bp) vybrány pro sekvenování. Celkem bylo osekvenováno 60 amplikonů. DNA sekvence byly porovnány s GenBank databází v BLAST vyhledávači. Několik sekvencí bylo navzájem homologních a tak konečné množství unikátních osekvenovaných amplikonů bylo 31 (Tabulka 2, str. P6). Pomocí metody kvantitativní RT-PCR jsme studovali expresi 5 vybraných genů po celé období preimplantačního vývoje: *centromere protein, 350/400 kDa (CENPF, mitosin), splicing factor arginine/serine-rich 3 (SRFS3), high mobility group nucleosomal binding domain 2 (HMGN2) protein* a eukaryotické translační iniciační faktory *EIF4A2* a *EIF4E*. *CENPF* má významnou roli v buněčném dělení, chromozomové kondenzaci, regulaci transkripce a průběhu buněčného cyklu (Holt et al., 2005; Zhou et al., 2005). Jeho transkript byl zvýšený od 2-buněčného do 4-buněčného stadia a později se zvyšoval v pozdním 8-buněčném stádiu. V pozdním 8-buněčném stádiu byla transkripce *CENPF* senzitivní k α-amanitinu, inhibitoru RNA polymerázy II, což potvrzuje jeho de novo transkripci v tomto stádiu (Fig2 A, str. P7). Exprese během období majoritní genomové aktivace souhlasí s důležitou rolí *CENPF* v mitotickém buněčném cyklu.

SRFS3 (SRp20) patří do proteinové rodiny SR faktorů, které se účastní sestřihu mRNA. Samotný SRFS3 se také podílí na exportu mRNA z jádra do cytoplazmy (Huang a Steitz, 2001) a regulaci alternativní polyadenylace (Lou et al., 1998). Mutantní myší embrya, která měla inaktivovaný gen *SRFS3* špatně formují blastocystu a většinou končí svůj vývoj ve stádiu moruly (Jumaa et al., 1999). Exprese transkriptu *SRFS3* se zvyšovala od 2-buněčného stadia do 4-buněčném stadia, pak klesala a k dalšímu zvýšení docházelo v pozdním 8-buněčném stádiu (Fig2 C, str. P7). Transkripce *SRFS3* byla α-amanitin senzitivní jak v 4-buněčném tak v 8-buněčném stádiu. Celkově můžeme shrnout, že exprese *SRFS3* vzrůstala během raného vývoje bovinního embrya na úrovních minoritní i majoritní genomové aktivace.

HMGN2 patří do skupiny proteinů s vysokou mobilitou, které hrají významnou roli při regulaci mnoha jaderných funkcí (Bustin, 2001; Agresti a Bianchi, 2003). Transkript *HMGN2* byl zvýšený ve 2-buněčném stádiu a ve stádiu moruly, ve které byla jeho transkripce senzitivní k α-amanitinu (Fig2 B, str. P7). Předpokládáme, že syntéza mRNA pro *HMGN2* v tomto období je následována překladem do proteinu, nutného pro modifikaci chromatinové struktury, která se tak stává transkripčně přístupnou.

Proteiny EIF4A2 a EIF4E jsou součástí translačního iniciačního komplexu EIF4F spolu s EIF4G1 (Raught a Gingras, 1999). Během raného vývoje ukazují stejný vzorec exprese, charakterizovaný nárůstem v období majoritní genomové aktivace.

Pro pět genů nalezených pomocí SSH byla prostudována jejich exprese během celého období preimplantačního vývoje u skotu. Všechny zkoumané geny hrají důležitou roli v raném vývoji embrya. U genu *SRFS3* jsme nalezli expresi během minoritní genomové aktivace, což potvrdila i citlivost jeho transkripce k α-amanitinu v tomto stádiu. Jako jediný gen z pěti studovaných genů tak potvrdil očekávané výsledky z SSH knihovny a byl zároveň prvním genem s významnou funkcí, jehož exprese byla nalezena již v průběhu minoritní genomové aktivace. I jiné studie využívající SSH pro studium preimplantačního vývoje ukazují značnou falešnou pozitivitu této metody a proto je nutné provádět další ověření metodou kvantitativní RT-PCR nebo používat SSH jen jako předstupeň pro selekci zajímavých transkriptů například pro přípravu mikročipů. Roli genu *CENPF* jsme v následující studii podrobně studovali pomocí specifického umlčení dsRNA. Důvodem výběru tohoto genu byl jednak jeho význam pro buněčné dělení, jednak dostupnost příslušné protilátky.

## 3.2. Toralova T, Susor A, Nemcova L, Kepkova K, Kanka J.: Silencing *CENPF* in bovine preimplantation embryo induces arrest at 8-cell stage. Reproduction. 2009 Nov;138(5):783-91. Epub 2009 Aug 3.

V naší předešlé studii jsme pomocí metody SSH identifikovali několik genů exprimovaných během časného preimplantačního vývoje bovinních embryí. Jedním z těchto genů byl *CENPF* (centromeric protein F, mitosin). CENPF hraje důležitou roli v dělení somatických buněk a jeho exprese a lokalizace je regulována v závislosti na stádiu buněčného cyklu. Deplece CENPF způsobuje v somatických buňkách poruchy správného seřazení chromozomů během mitózy a destabilizuje interakci kinetochor s mikrotubuly s následnou aneuploidií nebo zastavením buněčného dělení v metafázi (Bomont et al., 2005; Holt et al., 2005; Yang et al., 2005). Buněčný cyklus během preimplantačního vývoje savčích oocytů je značně odlišný od cyklu somatických buněk (zkrácená G1 fáze, naopak delší M fáze) a funkce genu *CENPF* během tohoto období zatím nebyla studována. Vzhledem k jeho expresi v období majoritní genomové aktivace (8-buněčné stádium), zaměřili jsme se v naší studii na osud bovinního embrya po umlčení *CENPF* pomocí injikace double-strand RNA (dsRNA) do embrya ve stádiu zygoty.

Mikroinjekce *CENPF* dsRNA efektivně a specificky způsobuje degradaci mRNA *CENPF*. V pozdním 8-buněčném stádiu byla mRNA redukována o 96% v porovnání s neinjikovanými kontrolami a o 94,9% ve srovnání s kontrolami injikovanými green fluorescent proteine (*GFP*) dsRNA. V pozdním 16-buněčném stádiu byla mRNA redukována o 97,8% v porovnání s neinjikovanými kontrolami a o 98,5% ve srovnání s *GFP* dsRNA injikovanými kontrolami (Fig. 1., str. P14). Nebyl nalezený žádný rozdíl v množství *CENPF* mRNA mezi neinjikovanými kontrolami a *GFP* dsRNA injikovanými kontrolami.

Dále jsme sledovali účinek *CENPF* dsRNA injikace na hladinu příslušného proteinu pomocí imunofluorescenčního značení polyklonální anti-CENPF protilátkou specifickou pro C-konec proteinu (Fig. 3., str. P15). U neinjikovaných a GFP dsRNA injikovaných kontrolních embryí byl CENPF jasně kolokalizován s jádry blastomer, zatímco v *CENPF* dsRNA injikovaných embryích nebyla detekována podobná lokalizace a úroveň fluorescence byla velmi nízká.

Pro zjištění účinku deplece *CENPF* mRNA na vývojovou kompetenci embrya byla monitorována embrya zastavená v jednotlivých vývojových stádiích. V období od 2-buněčného stádia až do EGA (8-buněčné stádium) nebylo pozorováno žádné snížení vývojové kompetence. Signifikantně nižší množství *CENPF* dsRNA injikovaných 8-buněčných embryí bylo schopno vývoje do 16 buněk nebo dále v porovnání s kontrolními skupinami, které měly i lepší morfologickou kvalitu (Fig. 4., str. P16). Nejčetnější defekt *CENPF* dsRNA injikovaných embryí byla nestejná velikost blastomer a částečná segregace cytoplazmy. Imunofluorescence odhalila u *CENPF* dsRNA injikovaných embryí fragmentovaná nebo úplně chybějící jádra v části buněk (Fig. 3C, str. P15). To naznačuje, že v části blastomer je zastaven postup buněčným cyklem, zatímco některé blastomery jsou schopné tento blok překonat a dále se vyvíjet. K

zjištění, zda je CENPF protein cyklicky degradován a resyntetizován v pre-EGA embryích, jsme použili inhibitor cycloheximid (CHX) k blokování proteinové syntézy během kultivace od pozdního 4-buněčného stádia do 8-buněčného stádia a od pozdního 8-buněčného stádia do 16-buněčného stádia. Následně byla embrya zkoumána pomocí imunofluorescence pro přítomnost CENPF. V kultivaci s CHX od pozdního 4-buněčného stádia do 8-buněčného stádia nebyl pozorován žádný signifikantní rozdíl v intenzitě fluorescence, což naznačuje, že CENPF není degradována před EGA. Oproti tomu výsledky kultivace s CHX od pozdního 8-buněčného stádia do 16-buněčného stádia vykazovaly výrazně sníženou intenzitu fluorescence při barvení anti-CENPF protilátkou a signál nebyl detekován ve všech jádrech. Přesto kompletní degradace CENPF nebyla pozorována (Fig. 6, str. P17). S výsledky, získanými pomocí kultivace v CHX, souhlasí naše předešlá studie (Kanka et al., 2009), kde jsme prokázali aktivaci transkripce *CENPF* v pozdním 8-buněčném stádiu. Zastavení embryí s vyčerpanou zásobou *CENPF* mRNA právě v 8-buněčném stádiu naznačuje, že do této doby embryo užívá maternální protein.

Pomocí imunofluorescenční analýzy jsme dále monitorovali lokalizaci CENPF během buněčného cyklu v bovinních embryích po EGA. Získaná data potvrzují stejnou expresi a lokalizaci, jaká byla pozorována u humánního proteinu v somatických buňkách. Nejvíce blastomer analyzovaných embryí bylo v interfázi. V souladu s pozorováním na somatických buňkách (Liao et al., 1995; Zhu et al., 1995; Hussein a Taylor, 2002; Feng et al., 2006), byl CENPF detekován v celém jádře kromě jadérka. S postupující kondenzací se na chromozomech objevují fluorescenční body (Fig. 7, str. P18), což je konzistentní s kinetochorovou lokalizací během profáze a prometafáze v somatických buňkách (Liao et al., 1995; Zhu et al., 1995; Hussein a Taylor, 2002; Feng et al., 2006). Na konci mitózy přestává být CENPF imunofluorescencí detekovatelný, což koresponduje s degradací CENPF v somatických buňkách (Liao et al., 1995; Zhu et al., 1995; Hussein a Taylor, 2002; Feng et al., 2006).

Naše výsledky potvrzují nezbytnost exprese *CENPF* pro správný průběh preimplantačního vývoje v období po majoritní genomové aktivaci.

### 3.3. Katerina Vodickova Kepkova, Petr Vodicka, Tereza Toralova, Miloslava Lopatarova, Svatopluk Cech, Radovan Dolezel, Vitezslav Havlicek, Urban Besenfelder, Anna Kuzmany, Marc-Andre Sirard, Jozef Laurincik, Jiri Kanka.: Transcriptomic analysis of *in vivo* and *in vitro* produced bovine embryos revealed a developmental change in *cullin 1* expression during maternal-to-embryonic transition. Theriogenology, accepted for publication.

V této publikaci jsme se zaměřili na srovnání transkriptomu *in vivo* získaných a *in vitro* produkovaných bovinních embryí v dvou kritických obdobích preimplantačního vývoje, tedy ve stádiu 4 buněk (minoritní genomová aktivace) a 8 buněk (majoritní genomová aktivace). Cílem tohoto srovnání bylo nalézt geny, jejichž exprese se mění během preimplantačního vývoje skotu v závislosti na prostředí a které tak mohou souviset s kvalitou embrya.

Studie se skládala z několika zásadních úkolů. Prvním z nich byla příprava cDNA knihoven obohacených o embryonální transkripty. Pomocí série subtraktivních hybridizací (SSH) v následujícím uspořádání: 4-buněčné stádium – oocyty v MII fázi (Kanka et al., 2009), 8-buněčné stádium – 4-buněčné stádium a 4-buněčné stádium – 8-buněčné stádium byly připraveny tři cDNA knihovny. Tyto cDNA knihovny byly odeslány na Université Laval, Québec, kde se staly součástí specifického bovinního preimplantačního mikročipu (BlueChip) (Sirard et al., 2005). Pomocí tohoto mikročipu jsme pak v naší laboratoři charakterizovali změny v profilu genové exprese způsobené *in vitro* kultivací a to tak, že v prvním experimentu byla pomocí dvoukanálové mikročipové analýzy přímo srovnána 4-buněčná *in vivo* získaná a *in vitro* produkovaná embrya a v druhém experimentu přímo srovnána 8-buněčná *in vivo* získaná a *in vitro* produkovaná embrya. Kompletní data z mikročipové analýzy jsou přístupná v databázi Gene Expression Omnibus (GEO) pod přístupovým číslem **GSE24714**. Analýza rozdílů genové exprese pomocí SAM (significance analysis of microarrays) softwaru (Tusher et al., 2001) odhalila změnu ve 134 transkriptech mezi *in vitro* embryi kultivovanými v COOK BVC/BVB médiu a *in vivo* embryi ve 4-buněčném stádiu a 97 rozdílně exprimovaných transkriptů mezi 8-buněčnými *in vitro* kultivovanými a *in vivo* získanými embryi.

Následně jsme k identifikovaným rozdílně exprimovaným transkriptům nalezli jejich lidské ortology (tam kde to bylo možné) a příslušná SwisProt přístupová čísla jsme použili pro vyhledání možných interagujících proteinů v databázi Interlogous Interaction Database (ID2). Model protein-proteinové interakční sítě (Fig. 1., str. P48, zjednodušený pohled) byl na základě této databáze zobrazen pomocí softwaru NAViGaTOR (Brown a Jurisica, 2005). V takto modelované interakční síti byly identifikovány čtyři navzájem vysoce propojené skupiny proteinů (kliky). Ze tří z těchto skupin bylo vybráno po jednom genu pro další studium jejich exprese během preimplantačního vývoje pomocí kvantitativní RT-PCR (*FBL, CNOT4, CUL1*). Další čtyři geny byly pro podrobnou studii vybrány ze skupiny rozdílně regulovaných transkriptů na základě jejich možné důležitosti pro preimplantační vývoj (*BUB3, NOLC1, PCAF, GABPA*).

Transkript genu *cullin 1-like* (*CUL1*) byl identifikován jako více zastoupený v *in vitro* produkovaných embryích jak ve stádiu 4 tak 8 buněk. U mnohobuněčných živočichů bylo

popsáno pět hlavních skupin cullinů (CUL1-CUL5). Cullin 1 tvoří ubikvitin-ligázový komplex SCF, který se skládá ze tří stálých komponent, Skp1, CUL1 a Rbx1 a variabilní komponenty, F-box proteinu, který reguluje substrátovou specifitu. SCF komplex zajišťuje ubikvitinaci proteinů důležitých pro průchod buněčným cyklem, zejména během G1/S přechodu a je také důležitý pro regulaci duplikace centrosomu (Freed et al., 1999). Při qRT-PCR s primery navrženými k identifikaci bovinního *CUL1* jsme identifikovali dva produkty stejné délky, ale s různou teplotou meltingu. První produkt byl detekován od stádia MII oocytů až po časné 8-buněčné stádium, zatímco druhý produkt byl nalezen v embryích od pozdního 8-buněčného stádia až do blastocysty (Fig. 2A, str. P49). Sekvenování obou produktů potvrdilo, že náleží dvěma různým genům z cullinové rodiny, (UniGene IDs Bt.36789, respektive Bt.6490), které leží v různých úsecích chromozómu 4. Transkript přepisovaný v MII oocytech až časných 8-buněčných embryích byl identifikován jako *cullin 1-like* transcript variant 1 (GeneBank ID XM_589507), transkript přítomný od 8-buněčného stádia do blastocysty byl identifikován jako *cullin 1,* transcript variant 3 mRNA (GeneBank ID XM_876699) (Fig. 2B, str. P49). Detekce CUL1 proteinu pomocí imunofluorescence neprokázala změny v jeho množství v průběhu sledovaného období (MII oocyt-morula, Fig. 2C, str. P49). Tyto výsledky naznačují, že *cullin 1-like* představuje maternální transkript, který je postupně degradován po fertilizaci a nahrazen transkriptem *cullin 1* v období aktivace embryonálního genomu. Význam této změny v expresi zatím není známý.

*Fibrillarin* (*FBL*) byl identifikován jako více zastoupený v *in vitro* produkovaných 4 buněčných embryích. Fibrillarin je lokalizován ve fibrilárních centrech a denzní fibrilární komponentě jadérka a podílí se na zpracování primárních rRNA transkriptů. Bylo prokázáno, že de novo syntéza fibrillarinu je nutná pro znovuzformování funkčních jadérek během období majoritní genomové aktivace u skotu (Svarcova et al., 2007). Nově jsme detekovali protein fibrillarin pomocí imunofluorescence v raných 8-buněčných embryích (Fig. 3, str. P50). Tato detekce koreluje se vzrůstajícím transkriptem (Fig. 4A, str. P51).

Analýza pomocí metody qRT-PCR odhalila významné rozdíly v hladině transkriptů mezi *in vitro* a *in vivo* získanými embryi v pozdních 8-buněčných stádiích u genů *BUB3, NOLC1, PCAF, GABPA* a *CNOT4* (Fig. 4B-F, str. P51). Celkově naše studie pomocí mikročipové analýzy, qRT-PCR a imunofluorescence identifikovala změny v expresi několika genů v časném preimplantačním vývoji skotu. Zejména změna z maternální exprese genu *cullin 1-like* na embryonální expresi genu *cullin 1* zatím nebyla nikým popsána a plánujeme ji dále studovat.

Soubor nalezených genů představuje pracovní nástroj, umožňující pokládat otázky, související s normálním vývojem embryí v podmínkách *in vivo*. Tento soubor genů může také sloužit jako ukazatel kvality embryí v budoucí smaze optimalizovat podmínky kultivace *in vitro*.

# 4. ZÁVĚRY

- **Metoda SSH byla použita k vytvoření cDNA knihovny obohacené o transkripty syntetizované ve stádiu minoritní genomové aktivace. Embrya ve 4-buněčném stádiu byla použita jako tester oproti oocytům v MII fázi. Celkem bylo osekvenováno 60 amplikonů, z toho unikátních sekvencí bylo 31. Z těchto sekvencí jsme vybrali 5 genů: *centromere protein, 350/400 kDa (CENPF, mitosin), splicing factor arginine/serine-rich 3 (SRFS3), high mobility group nucleosomal binding domain 2 (HMGN2) protein* a eukaryotické translační iniciační faktory *EIF4A2* a *EIF4E* pro následné ověření pomocí metody kvantitativní RT-PCR. Všechny zkoumané geny hrají důležitou roli v raném vývoji embrya.**
- **cDNA knihovna (4-buněčné stádium – MII oocty) byla použita jako součást specifického preimplantačního mikročipu (BlueChip), který byl připraven ze stádii bovinního preimplantačního vývoje v laboratoři Centre de Recherche en Biologie de la Reproduction, Département des Sciences Animales, Université Laval, Sainte-Foy, Québec, Canada.**
- **Pomocí metody SSH jsme připravili 2 cDNA knihovny. Jako tester bylo použito 4-buněčné stádium a jako driver 8-buněčné stádium a naopak (4-buněčné stádium – 8-buněčné stádium). Tyto dvě knihovny jsou také součástí expresního cDNA mikročipu (BlueChip).**
- **U genu *SRFS3* bylo prokázáno, že je exprimován během minoritní genomové aktivace, což potvrdila i citlivost jeho transkripce k α-amanitinu v tomto stádiu.**
- **Mikroinjekce *CENPF* dsRNA efektivně a specificky způsobuje degradaci mRNA *CENPF*.**
- **Deplece *CENPF* mRNA měla významný vliv na vývojovou kompetenci embrya. Signifikantně nižší množství *CENPF* dsRNA injikovaných 8-buněčných embryí bylo schopno vývoje do 16 buněk nebo dále.**
- **V kultivaci s CHX pro blokování proteinové sytézy od pozdního 4-buněčného stádia do 8-buněčného stádia nebyl pozorován žádný signifikantní rozdíl v intenzitě fluorescence, což naznačuje, že CENPF není degradován před EGA. Oproti tomu výsledky kultivace s CHX od pozdního 8-buněčného stádia do 16-buněčného stádia vykazovaly výrazně sníženou intenzitu fluorescence při barvení anti-CENPF protilátkou a signál nebyl detekován ve všech jádrech, přestože kompletní degradace CENPF nebyla pozorována.**
- **Pro stanovení expresního rozdílu mezi bovinními 4-buněčnými embryi *in vitro* a *in vivo* a 8-buněčnými embryi *in vitro* a *in vivo* jsme použili specifický bovinní preimplantační cDNA mikročip (BlueChip). Mezi 4-buněčnými embryi bylo rozdílně exprimováno 134 genů a mezi 8-buněčnými embryi 97 genů. Analýza pomocí metody qRT-PCR odhalila**

**významné rozdíly v hladině transkriptů mezi *in vitro* a *in vivo* získanými embryi v pozdních 8-buněčných stádiích u genů *BUB3, NOLC1, PCAF, GABPA* a *CNOT4*.**

- **Pomocí imunofluorescence jsme nalezli nově syntetizovaný protein fibrillarin již ve stádiu raných 8 buněk po *in vitro* kultivaci. Tato detekce je potvrzena pomocí metody qRT-PCR na úrovni mRNA.**
- **U jednoho z genů, identifikovaného jako rozdílně exprimovaný pomocí mikročipové analýzy sondou proti *cullinu 1-like*, jsme metodou real-time RT-PCR a následnou sekvenací zjistili, že dochází k přepínaní exprese dvou příbuzných genů během preimplantačního vývoje. Zatímco v MII oocytech až raných 8-buněčných embryích je přítomen transkript pro *cullin 1-like*,transkripční variantu 1, v čase majoritní embryonální aktivace (ve stádiu 8 buněk) dochází k přepnutí na expresi transkriptu *cullin 1*, transkripční variaty 3 (od stádia pozdních 8 buněk do hatched blastocysty). U *in vivo* embryí k přechodu dochází ve stádiu mezi 8 – 16 buňkami.**

**Soubor nalezených genů představuje pracovní nástroj, umožňující pokládat otázky, související s normálním vývojem embryí v podmínkách *in vivo* a může sloužit jako ukazatel kvality embryí v budoucí snaze optimalizovat podmínky kultivace *in vitro*.**

# 5. CITOVANÁ LITERATURA

Adjaye J, Herwig R, Brink TC, Herrmann D, Greber B, Sudheer S, Groth D, Carnwath JW, Lehrach H, Niemann H (2007) Conserved molecular portraits of bovine and human blastocysts as a consequence of the transition from maternal to embryonic control of gene expression. Physiol. Genomics 31:315-327.

Agresti A, Bianchi ME (2003) HMGB proteins and gene expression. Curr. Opin. Genet. Dev 13:170-178.

Aoki F, Worrad DM, Schultz RM (1997) Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev. Biol 181:296-307.

Bachvarova RF (1992) A maternal tail of poly(A): the long and the short of it. Cell 69:895-897.

Becker DL, Leclerc-David C, Warner A (1992) The relationship of gap junctions and compaction in the preimplantation mouse embryo. Dev. Suppl:113-118.

Betts DH, King WA (2001) Genetic regulation of embryo death and senescence. Theriogenology 55:171-191.

Bilodeau-Goeseels S, Schultz GA (1997) Changes in ribosomal ribonucleic acid content within in vitro-produced bovine embryos. Biol. Reprod 56:1323-1329.

Bloor DJ, Wilson Y, Kibschull M, Traub O, Leese HJ, Winterhager E, Kimber SJ (2004) Expression of connexins in human preimplantation embryos in vitro. Reprod. Biol. Endocrinol 2:25.

Bomont P, Maddox P, Shah JV, Desai AB, Cleveland DW (2005) Unstable microtubule capture at kinetochores depleted of the centromere-associated protein CENP-F. EMBO J 24:3927-3939.

Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA (1982) Normal development following in vitro fertilization in the cow. Biol. Reprod 27:147-158.

Brevini TAL, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F (2002) Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. Mol. Reprod. Dev 63:510-517.

Brown KR, Jurisica I (2005) Online predicted human interaction database. Bioinformatics 21:2076-2082.

Bui LC, Léandri RD, Renard JP, Duranthon V (2005) SSH adequacy to preimplantation mammalian development: scarce specific transcripts cloning despite irregular normalisation. BMC Genomics 6:155.

Bustin M (2001) Chromatin unfolding and activation by HMGN(*) chromosomal proteins. Trends Biochem. Sci 26:431-437.

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol 25:169-193.

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR - a perspective. J Mol Endocrinol 34:597-601.

Cai X, Hagedorn CH, Cullen BR (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10:1957-1966.

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques 37:112-114, 116, 118-119.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. U.S.A 93:6025-6030.

Dode MAN, Dufort I, Massicotte L, Sirard M-A (2006) Quantitative expression of candidate genes for developmental competence in bovine two-cell embryos. Molecular Reproduction and Development 73:288-297.

Edwards RG (2003) Aspects of the molecular regulation of early mammalian development. Reprod. Biomed. Online 6:97-113.

Fair T, Murphy M, Rizos D, Moss C, Martin F, Boland MP, Lonergan P (2004) Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. Mol. Reprod. Dev 67:136-144.

Feng J, Huang H, Yen TJ (2006) CENP-F is a novel microtubule-binding protein that is essential for kinetochore attachments and affects the duration of the mitotic checkpoint delay. Chromosoma 115:320-329.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806-811.

Freed E, Lacey KR, Huie P, Lyapina SA, Deshaies RJ, Stearns T, Jackson PK (1999) Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. Genes Dev 13:2242-2257.

Frei RE, Schultz GA, Church RB (1989) Qualitative and quantitative changes in protein synthesis occur at the 8-16-cell stage of embryogenesis in the cow. J. Reprod. Fertil 86:637-641.

Fulka H, St John JC, Fulka J, Hozák P (2008) Chromatin in early mammalian embryos: achieving the pluripotent state. Differentiation 76:3-14.

Hamatani T, Carter MG, Sharov AA, Ko MSH (2004) Dynamics of global gene expression changes during mouse preimplantation development. Dev. Cell 6:117-131.

He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet 5:522-531.

Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N.Y.) 11:1026-1030.

t Hoen PAC, de Kort F, van Ommen GJB, den Dunnen JT (2003) Fluorescent labelling of cRNA for microarray applications. Nucleic Acids Research 31:e20.

Holt SV, Vergnolle MAS, Hussein D, Wozniak MJ, Allan VJ, Taylor SS (2005) Silencing Cenp-F weakens centromeric cohesion, prevents chromosome alignment and activates the spindle checkpoint. J. Cell. Sci 118:4889-4900.

Huang Y, Steitz JA (2001) Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. Mol. Cell 7:899-905.

Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6:279-284.

Hussein D, Taylor SS (2002) Farnesylation of Cenp-F is required for G2/M progression and degradation after mitosis. J Cell Sci 115:3403-3414.

Jumaa H, Wei G, Nielsen PJ (1999) Blastocyst formation is blocked in mouse embryos lacking the splicing factor SRp20. Curr. Biol 9:899-902.

Kanka J (2003) Gene expression and chromatin structure in the pre-implantation embryo. Theriogenology 59:3-19.

Kanka J, Kepková K, Nemcová L (2009) Gene expression during minor genome activation in preimplantation bovine development. Theriogenology 72:572-583.

Katz-Jaffe MG, McCallie BR, Preis KA, Filipovits J, Gardner DK (2009) Transcriptome analysis of in vivo and in vitro matured bovine MII oocytes. Theriogenology 71:939-946.

Ko MSH (2004) Embryogenomics of pre-implantation mammalian development: current status. Reprod. Fertil. Dev 16:79-85.

Kues WA, Sudheer S, Herrmann D, Carnwath JW, Havlicek V, Besenfelder U, Lehrach H, Adjaye J, Niemann H (2008) Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo. Proc. Natl. Acad. Sci. U.S.A 105:19768-19773.

Laurincik J, Hyttel P, Baran V, Eckert J, Lucas-Hahn A, Pivko J, Niemann H, Brem G, Schellander K (1998) A detailed analysis of pronucleus development in bovine zygotes in vitro: cell-cycle chronology and ultrastructure. Mol. Reprod. Dev 50:192-199.

Lee SB, Esteban M (1994) The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology 199:491-496.

Lequarre AS, Marchandise J, Moreau B, Massip A, Donnay I (2003) Cell cycle duration at the time of maternal zygotic transition for in vitro produced bovine embryos: effect of oxygen tension and transcription inhibition. Biol. Reprod 69:1707-1713.

Liao H, Winkfein RJ, Mack G, Rattner JB, Yen TJ (1995) CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. J. Cell Biol 130:507-518.

Liu Z, Foote RH (1997) Effects of amino acids and alpha-amanitin on bovine embryo development in a simple protein-free medium. Mol. Reprod. Dev 46:278-285.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Lonergan P, Gutiérrez-Adán A, Pintado B, Fair T, Ward F, Fuente JD, Boland M (2000) Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro. Mol. Reprod. Dev 57:146-152.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP (1999) Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. J. Reprod. Fertil 117:159-167.

Lonergan P, Rizos D, Kanka J, Nemcova L, Mbaye AM, Kingston M, Wade M, Duffy P, Boland MP (2003) Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. Reproduction 126:337-346.

Lonergan P, Fair T, Corcoran D, Evans ACO (2006) Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. Theriogenology 65:137-152.

Lou H, Neugebauer KM, Gagel RF, Berget SM (1998) Regulation of alternative polyadenylation by U1 snRNPs and SRp20. Mol. Cell. Biol 18:4977-4985.

Marmorstein R (2001) Protein modules that manipulate histone tails for chromatin regulation. Nat. Rev. Mol. Cell Biol 2:422-432.

Meirelles FV, Caetano AR, Watanabe YF, Ripamonte P, Carambula SF, Merighe GK, Garcia SM (2004) Genome activation and developmental block in bovine embryos. Anim. Reprod. Sci 82-83:13-20.

Memili E, Dominko T, First NL (1998) Onset of transcription in bovine oocytes and preimplantation embryos. Mol. Reprod. Dev 51:36-41.

Memili E, First NL (1998) Developmental changes in RNA polymerase II in bovine oocytes, early embryos, and effect of alpha-amanitin on embryo development. Mol. Reprod. Dev 51:381-389.

Memili E, First NL (1999) Control of gene expression at the onset of bovine embryonic development. Biol. Reprod 61:1198-1207.

Memili E, First NL (2000) Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. Zygote 8:87-96.

Misirlioglu M, Page GP, Sagirkaya H, Kaya A, Parrish JJ, First NL, Memili E (2006) Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. Proc. Natl. Acad. Sci. U.S.A 103:18905-18910.

Mohan M, Hurst AG, Malayer JR (2004) Global gene expression analysis comparing bovine blastocysts flushed on day 7 or produced in vitro. Mol. Reprod. Dev 68:288-298.

Mohan M, Ryder S, Claypool PL, Geisert RD, Malayer JR (2002) Analysis of gene expression in the bovine blastocyst produced in vitro using suppression-subtractive hybridization. Biol. Reprod 67:447-453.

Muller PY, Janovjak H, Miserez AR, Dobbie Z (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques 32:1372-1374, 1376, 1378-1379.

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Meth. Enzymol 155:335-350.

Nganvongpanit K, Müller H, Rings F, Gilles M, Jennen D, Hölker M, Tholen E, Schellander K, Tesfaye D (2006)(a) Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. Mol. Reprod. Dev 73:153-163.

Nganvongpanit K, Müller H, Rings F, Hoelker M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Schellander K, Tesfaye D (2006)(b) Selective degradation of maternal and embryonic transcripts in in vitro produced bovine oocytes and embryos using sequence specific double-stranded RNA. Reproduction 131:861-874.

Niemann H, Wrenzycki C (2000) Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. Theriogenology 53:21-34.

Nygaard V, Hovig E (2006) Options available for profiling small samples: a review of sample amplification technology when combined with microarray profiling. Nucleic Acids Res 34:996-1014.

Paradis F, Vigneault C, Robert C, Sirard M-A (2005) RNA interference as a tool to study gene function in bovine oocytes. Mol. Reprod. Dev 70:111-121.

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36.

Raught B, Gingras AC (1999) eIF4E activity is regulated at multiple levels. Int. J. Biochem. Cell Biol 31:43-57.

Ritz C, Spiess A-N (2008) qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. Bioinformatics 24:1549-1551.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev 61:234-248.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sandy P, Ventura A, Jacks T (2005) Mammalian RNAi: a practical guide. BioTechniques 39:215-224.

Sarmento OF, Digilio LC, Wang Y, Perlin J, Herr JC, Allis CD, Coonrod SA (2004) Dynamic alterations of specific histone modifications during early murine development. J. Cell. Sci 117:4449-4459.

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467-470.

Schier AF (2007) The maternal-zygotic transition: death and birth of RNAs. Science 316:406-407.

Schinke-Braun M, Couget JA (2007) Expression profiling using affymetrix genechip probe arrays. Methods Mol. Biol 366:13-40.

Schultz GA, Heyner S (1992) Gene expression in pre-implantation mammalian embryos. Mutation Research/Reviews in Genetic Toxicology 296:17-31.

Schultz RM (2002) The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum. Reprod. Update 8:323-331.

Sirard M-A, Dufort I, Vallée M, Massicotte L, Gravel C, Reghenas H, Watson AJ, King WA, Robert C (2005) Potential and limitations of bovine-specific arrays for the analysis of mRNA levels in early development: preliminary analysis using a bovine embryonic array. Reprod Fertil Dev 17:47-57

Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3:Article3.

Smyth GK, Speed T (2003) Normalization of cDNA microarray data. Methods 31:265-273.

Stanton J-AL, Macgregor AB, Green DPL (2003) Gene expression in the mouse preimplantation embryo. Reproduction 125:457-468.

Steptoe PC, Edwards RG (1978) BIRTH AFTER THE REIMPLANTATION OF A HUMAN EMBRYO. The Lancet 312:366.

Steuerwald N, Cohen J, Herrera RJ, Brenner CA (1999) Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. Mol. Hum. Reprod 5:1034-1039.

Subkhankulova T, Livesey FJ (2006) Comparative evaluation of linear and exponential amplification techniques for expression profiling at the single-cell level. Genome Biol 7:R18.

Svarcova O, Laurincik J, Avery B, Mlyncek M, Niemann H, Maddox-Hyttel P (2007) Nucleolar development and allocation of key nucleolar proteins require de novo transcription in bovine embryos. Mol. Reprod. Dev 74:1428-1435.

Svoboda P, Stein P, Hayashi H, Schultz RM (2000) Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development 127:4147-4156.

Telford NA, Watson AJ, Schultz GA (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol. Reprod. Dev 26:90-100.

Tomek W, Torner H, Kanitz W (2002) Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro. Reprod. Domest. Anim 37:86-91.

Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U.S.A 98:5116-5121.

Vallée M, Robert C, Méthot S, Palin M-F, Sirard M-A (2006) Cross-species hybridizations on a multi-species cDNA microarray to identify evolutionarily conserved genes expressed in oocytes. BMC Genomics 7:113.

Van Soom A, Boerjan ML, Bols PE, Vanroose G, Lein A, Coryn M, de Kruif A (1997) Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. Biol. Reprod 57:1041-1049.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.

Wassarman PM, Kinloch RA (1992) Gene expression during oogenesis in mice. Mutation Research/Reviews in Genetic Toxicology 296:3-15.

Watson AJ, Barcroft LC (2001) Regulation of blastocyst formation. Front. Biosci 6:D708-730.

Watson AJ, Natale DR, Barcroft LC (2004) Molecular regulation of blastocyst formation. Anim. Reprod. Sci 82-83:583-592.

Watson AJ, Westhusin ME, De Sousa PA, Betts DH, Barcroft LC (1999) Gene expression regulating blastocyst formation. Theriogenology 51:117-133.

Wianny F, Zernicka-Goetz M (2000) Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell Biol 2:70-75.

Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 22:130-131, 134-138.

Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. BioTechniques 39:75-85.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H (1996) Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived in vitro or in vivo. J. Reprod. Fertil 108:17-24.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H (1999) Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. Mol. Reprod. Dev 53:8-18.

Wrenzycki C, Herrmann D, Niemann H (2003) Timing of blastocyst expansion affects spatial messenger RNA expression patterns of genes in bovine blastocysts produced in vitro. Biol. Reprod 68:2073-2080.

Yang Z, Guo J, Chen Q, Ding C, Du J, Zhu X (2005) Silencing mitosin induces misaligned chromosomes, premature chromosome decondensation before anaphase onset, and mitotic cell death. Mol. Cell. Biol 25:4062-4074.

Yokoi H, Natsuyama S, Iwai M, Noda Y, Mori T, Mori KJ, Fujita K, Nakayama H, Fujita J (1993) Non-radioisotopic quantitative RT-PCR to detect changes in mRNA levels during early mouse embryo development. Biochem. Biophys. Res. Commun 195:769-775.

Zeng Y, Yi R, Cullen BR (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proc. Natl. Acad. Sci. U.S.A 100:9779-9784.

Zhou X, Wang R, Fan L, Li Y, Ma L, Yang Z, Yu W, Jing N, Zhu X (2005) Mitosin/CENP-F as a negative regulator of activating transcription factor-4. J. Biol. Chem 280:13973-13977.

Zhu X, Mancini MA, Chang KH, Liu CY, Chen CF, Shan B, Jones D, Yang-Feng TL, Lee WH (1995) Characterization of a novel 350-kilodalton nuclear phosphoprotein that is specifically involved in mitotic-phase progression. Mol. Cell. Biol 15:5017-5029.

# 6. SEZNAM PŘEDKLÁDANÝCH PUBLIKACÍ

**Gene expression during minor genome activation in preimplantation bovine development.**

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**Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage**

Toralová T, Susor A, Nemcová L, **Kepková K**, Kanka J.

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**Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition.**

**Katerina Vodickova Kepkova**, Petr Vodicka, Tereza Toralova, Miloslava Lopatarova, Svatopluk Cech, Radovan Dolezel, Vitezslav Havlicek, Urban Besenfelder, Anna Kuzmany, Marc-Andre Sirard, Jozef Laurincik, Jiri Kanka

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I. Petrovicova, F. Strejcek, O. Østrup, A. Lucas-Hahn, **K. Kepkova**, H. Niemann, J. Laurincik, P. Maddox-Hyttel

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# Gene expression during minor genome activation in preimplantation bovine development

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**Abstract**

The main goal of this study was to identify mRNA transcripts whose content increases during bovine minor embryonic genome activation. We compared the gene expression profile of the bovine 4-cell-stage embryo and MII oocyte using the technique of suppression subtractive hybridization. Differentially expressed amplicons were subcloned, and 60 of them were sequenced. The resulting DNA sequences were compared with GenBank databases using BLAST search. The expression of five differentially expressed genes with an apparent function in cell cycle progression, chromatin remodeling, and splicing or translation initiation was further characterized by a real-time RT-PCR. Centromere protein F, 350/400 ka (CENPF), and splicing factor arginine/serine-rich 3 (SRFS3) show an increase in mRNA content during the 2- to 4-cell and late 8-cell stages. For the high mobility group nucleosomal binding domain 2 (HMGN2), the level of mRNA increases in 2- to 4-cell and morula embryos. The transcription of splicing factor SRFS3 is α-amanitin sensitive both during 4-cell and late 8-cell stages. The transcription of CENPF and HMGN2 is α-amanitin sensitive only at late 8-cell stage and morula, respectively. SRFS3 represents the first described gene with an important function in preimplantation development, which is also expressed during bovine minor genome activation, and it is α-amanitin sensitive during this period. All described genes can play an important role in the preimplantation development of bovine embryos.


*Keywords:* Developmental biology; Embryo; Gene expression; Real-time RT-PCR; SSH

## 1. Introduction

The preimplantation development of mammals is characterized by transition from oogenetic to embryonic genomic control (embryonic genome activation; EGA). As development proceeds after fertilization, maternally inherited mRNA molecules decay and embryogenesis becomes dependent on the expression of genetic information derived from the embryonic genome [1–4].

The most detailed information on this topic was obtained from a mouse system. An early experiment using metabolic labeling to detect poly (A)+ and poly (A)– RNA synthesis revealed transcription at the 2-cell stage [5]. In keeping with this idea, a synthesis of heat shock protein, HSP70.1 (previously known as HSP 68 and HSP 70), was found [6,7]. Its mRNA synthesis was inhibited by α-amanitin (RNA polymerase II inhibitor). Based on these results, it was believed that first gene activation in the mouse started at the 2-cell stage.

The continuing development of techniques allowed the method of nucleus transfer to be applied to these studies. After the transfer of nuclei from the inner cell mass and somatic cells, 1-cell-stage mouse cytoplasm

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proved to be permissive for transcription [8]. With the incorporation of 5-bromouridine-5′-triphosphate (BrUTP), transcription was proved as early as at the late 1-cell stage [9] and even in the mid-S phase in the 1-cell-stage mouse embryo [10]. Studies on transgenic mice have also shown that specific transgenes are transcribed at the late 1-cell stage [11]. Although the 1-cell embryo is transcriptionally active, no α-amanitin sensitive transcript was detected using Affymetrix GeneChip, Santa Clara, CA [12]. Erv4 (previously known as MuERV–L) remains the only de novo transcript detected in 1-cell mouse embryos [13]. All these studies support the hypothesis that genome activation occurs in a stepwise manner in the mouse embryo [2].

It has been suggested that first genome activation is global and relatively promiscuous, and that the function of the subsequent transcriptionally repressive state is to dictate the appropriate profile of gene expression [14]. Using microarrays, global changes in gene expression were characterized during preimplantation mouse development [15]. The analyses revealed changes in the transcript profile in the 2-cell embryo, which were greater than previously recognized. Gene activation in the 2-cell-stage embryo may not be as global and promiscuous as previously proposed because the genes involved in cell proliferation, mitotic cell cycle, regulation of transcription, and DNA and protein metabolism are preferentially expressed [15]. Patterns of maternal RNA degradation and zygotic gene activation, including two major transient waves of de novo transcription, were described during mouse preimplantation development [16]. The first wave corresponds with zygotic genome activation (ZGA), which appeared between the late 1-cell-stage and early 2-cell-stage mouse embryo. Another study using microarrays also revealed dynamic patterns of gene activity up to the 2-cell stage [17]. The authors also support the possibility that ZGA occurs in a stepwise manner.

Although the successful execution of EGA is essential for further development, knowledge of specific mechanisms for initiating EGA is lacking. Changes were analyzed in polysomal maternal mRNAs (e.g., mRNAs that are actively translated between the mouse oocyte and 1-cell-stage embryo) [18]. Changes were found not only in a number of preferentially translated maternal mRNAs but also in functional classes of proteins encoded by these mRNAs. Maternal mRNAs expressed or enriched in 1-cell embryos contribute more to metabolic processes than do those in oocytes, which ensure homeostasis. The recruitment of maternal mRNAs after fertilization, prior to first cleavage, supports the successful initialization of EGA. It has been suggested that the recruitment of maternal cyclin A2 (Ccna2) mRNA leads to cyclin dependent kinase (Cdk2) activation [19]. CCNA2-CDK2 regulates the expression of numerous genes by regulating SP1 activity [20]. Recruiting maternal Ccna2 mRNA can modulate the function of proteins involved in the expression of large numbers of genes. Subsequently, EGA and a reprogramming of gene expression in mouse embryos may ensue.

Chromatin remodeling after fertilization is another fundamental event for transcriptional activation. Maternal BRG1, a catalytic subunit of the SWI/SNF related chromatin remodeling complex, unpacks the nucleosomes and enables binding of RNA polymerase II. Mouse embryos derived from eggs depleted in Brg1 have reduced transcriptional activity [21].

In contrast, the situation during transition from oogenetic to embryonic control is not well defined in large domestic mammals. In bovine embryos, embryonic genome activation appears at the 8- to 16-cell stage, as evidenced by [$^3$H]uridine incorporation (after short incubation) into nuclei and nucleoli at the 8-cell stage, major changes in the ultrastructure of blastomere nucleoli, and the pattern of protein synthesis [22,23]. The timing of EGA or the competence to sustain appreciable transcriptional activity in bovine embryos may be controlled temporally (i.e., at a time after fertilization), rather than by a developmental stage. Bovine embryos transfected with reporter genes at the 1-cell stage only express detectable amounts of reporter gene product after 42 h of culture at around the 4- to 8-cell stage or later. Reporter gene product was also detected in arrested 1-cell rabbit embryos at this time [24].

Studies using long-term exposure of 2- to 4-cell bovine embryos to [$^3$H]uridine followed by autoradiography revealed that transcriptional activity could be detected early in development [25]. Treatment with the transcriptional inhibitor α-amanitin blocked this incorporation. Bovine embryos labeled with $^{35}$S-UTP at the 2-cell stage revealed transcriptional activity [26]. The detection of [$^3$H]uridine incorporation into RNA showed that even 1-cell zygotes were transcriptionally active [27]. However, development can still proceed to the 8-cell stage in the presence of α-amanitin [28]. Recent advances in molecular technology facilitated studies of gene transcription in early embryos. Using differential display RT-PCR, α-amanitin sensitivity was first detected at the 2- to 5-cell stage but became predominant after the 6- to 8-cell stage of development [29,30]. Ribosomal RNA transcription visualized by in situ hybridization and subsequent silver staining of

argyrophilic nucleolar proteins was observed in the third cell cycle (4-cell stage) in a proportion of bovine embryos [31]. Global activation of the bovine embryonic genome at the 8-cell stage was studied using an Affymetrix bovine-specific DNA microarray [32]. In a similar study, the authors generated and characterized a cDNA library enriched in embryonic transcripts expressed at maternal to embryonic transition in bovine [33]. A high proportion of these genes are involved in gene transcription or RNA processing. Many of these transcripts are expressed in 6-cell to early 8-cell-stage embryos. All these findings follow similar observations in the mouse [2] and suggest there is a low level of transcriptional activity (so-called minor genome activation) between 1- and 4-cell-stage bovine embryos, followed by major genome activation at the 8-cell stage. However, the details are lacking, especially about specific genes activated during the minor genome activation period in farm animals.

The objective of this study was to identify a number of genes expressed during the minor genome activation period in preimplantation bovine embryos.

## 2. Materials and methods

### 2.1. Oocyte collection and in vitro fertilization

Bovine embryos were obtained after in vitro maturation of oocytes and their subsequent fertilization and culture in vitro [34]. In brief, follicles were dissected with fine scissors and then punctured. The oocytes were transferred to a modified Parker medium (MPM) [34] within 2 h of arrival at the laboratory. The oocytes were evaluated and selected according to cumulus morphology and transferred to 4-well Nunclon dishes (Nunc, Roskilde, Denmark) with 0.5 mL MPM and cultured in an atmosphere of 5% $CO_2$, 7% $O_2$, 88% $N_2$ at 39 °C for 24 h. Groups of 25 MII oocytes were washed with phosphate-buffered saline (PBS), immediately frozen, and stored at −80 °C until mRNA extraction.

For in vitro fertilization (IVF), cumulus-oocyte complexes were washed four times in PBS and once in fertilization medium, transferred in groups of up to 40 into 4-well dishes (Nunc) containing 250 μL fertilization medium [34] per well. Viable spermatozoa were washed in fertilization medium and pelleted by centrifugation at $100 \times g$ for 5 min. Spermatozoa were counted in a hemocytometer and diluted in the appropriate volume of fertilization medium to give a concentration of $2 \times 10^6$ spermatozoa/mL. A 250-μL aliquot of this suspension was added to each fertilization well to obtain a final concentration of $1 \times 10^6$ spermatozoa/mL. Plates were incubated for approximately 20 h at 39 °C in an atmosphere of 5% $CO_2$, 7% $O_2$, 88% $N_2$.

### 2.2. Embryo culture

At approximately 20 h postfertilization (hpf), presumptive zygotes were denuded by gentle pipetting and transferred to Bovine Vitro Cleave medium (BVC; Cook, Eight Mile Plains, Australia) and cultured in an atmosphere of 5% $CO_2$, 7% $O_2$, 88% $N_2$ at maximum humidity (25 zygotes in 25 μL medium under mineral oil). At 100 hpf, the BVC medium was replaced by Bovine Vitro Blast medium (BVB; Cook) and embryos were cultivated until the hatched blastocyst stage. The dishes were examined at 32, 44, 56, 92, 120, 156, and 180 hpf, and 2-cell, 4-cell, early 8-cell, late 8-cell embryos, morula, blastocysts, and hatched blastocysts were collected at each time point, respectively.

### 2.3. α-Amanitin treatment

To block RNA polymerase II dependent transcription, α-amanitin (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium at a final concentration of 100 μg/mL at the time of minor genome activation (from late 1-cell stage to 4-cell stage, 20 to 44 hpf) or at the time of major genome activation (from 4-cell stage to late 8-cell stage, 44 to 92 hpf, and in the HMGN2 study, from early 8-cell stage to morula stage, 62 to 110 hpf). After all α-amanitin treatments, embryos at the 4-cell stage (44 hpf), late 8-cell stage (92 hpf), and morula stage (110 hpf) were washed with PBS, immediately frozen, and stored at −80 °C. Control embryos were collected at the same time intervals as their treated counterparts, from the same fertilization/cultivation group, washed with PBS, immediately frozen, and stored at −80 °C. All pools were done in triplicate and contained 20 embryos.

### 2.4. RNA extraction

Poly (A)+ mRNA was extracted from pooled oocytes and embryos (25 in each group) using the Dynabeads mRNA DIRECT Micro Kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. RNA samples were stored at −80 °C.

### 2.5. Reverse transcription and Super SMART cDNA amplification

cDNA was synthesized from the extracted RNA and amplified using the Super SMART cDNA synthesis kit

(BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions.

### 2.6. Suppression subtractive hybridization (SSH)

Subtractive hybridization was performed using the Clontech PCR-Select cDNA subtraction kit (BD Biosciences Clontech), as previously described [35]. Subtraction was performed in one direction: 4-cell-stage cDNA served as the tester, and MII oocyte cDNA served as the driver. Four-cell-stage cDNA was chosen as the tester because we want to preferentially find genes exhibiting higher RNA content in this stage. In brief, both tester and driver cDNAs were digested with RsaI. Two different adaptors, namely, adaptor 1 or adaptor 2R, were ligated to two separate pools of tester cDNA in independent ligation reactions. Adaptor ligated tester cDNAs were then allowed to hybridize in the presence of excess driver cDNA in two separate hybridization reactions. The two tester populations ligated with the two different adaptors were then mixed in the presence of excess driver and allowed to hybridize for a second time. After the second hybridization, tester cDNA was subjected to primary and secondary PCR amplification. The products were analyzed by 2% agarose/ethidium bromide gel electrophoresis.

### 2.7. Cloning and sequencing of subtracted cDNA

Polymerase chain reaction products generated by SSH were subcloned into the pCR2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, CA, USA), and used to transform one shot chemically competent TOP 10 *Escherichia coli* cells. Colonies were grown for 16 h at 37 °C on Luria broth agar plates inoculated with 50 μg/mL kanamycin (Sigma-Aldrich) and 40 mg/mL X-Gal (Invitrogen) for blue-white colony selection.

Plasmids were extracted and inserts were sequenced using the Big Dye Terminator kit V.3.1 (Applied Biosystems, Foster City, CA, USA) and ABI Prism 310 (Applied Biosystems). The resulting sequences were identified using the Basic Local Alignment Search Tool (BLAST) [36].

### 2.8. mRNA isolation for quantitative real-time RT-PCR

All pools were done in triplicate and contained 20 oocytes or embryos from these different developmental stages: MII oocytes, 2-cell embryos, 4-cell embryos, early 8-cell embryos, late 8-cell embryos, morula embryos, blastocysts, and hatched blastocysts. Poly (A)+ mRNA was extracted from pooled oocytes and embryos (20 in each group) using the Dynabeads mRNA DIRECT Micro Kit (Dynal) according to the manufacturer's instructions. Luciferase mRNA (Promega, Madison, WI, USA) was added as an external mRNA standard (1 pg/1 oocyte or embryo) before isolation. The mRNA was eluted from Dynabeads in 20 μL 10 mM Tris-HCl after heating the samples at 85 °C for 2 min. Just before analysis, the volume was adjusted to 40 μL with RNase free $H_2O$.

### 2.9. Quantitative real-time RT-PCR

The expression of selected and control genes was estimated by a one-step RT-PCR with real-time detection. Experiments were carried out on Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) using a Qiagen OneStep RT-PCR Kit (Qiagen, Hilden, Germany). The 25 μL total reaction volume contained Qiagen OneStep RT-PCR Buffer (1x), dNTP Mix (400 μM of each), reverse and forward primers (both 400 μM), SybrGreenI (1:50.000 of 1000x stock solution; Molecular Probes, Eugene, OR, USA), RNasine inhibitor (Promega), Qiagen OneStep RT-PCR Enzyme Mix (1 μL), and template RNA (equivalent of 1 embryo). Reaction conditions were reverse transcription at 50 °C for 30 min, initial activation at 95 °C for 15 min; cycling: denaturation at 94 °C for 20 s, annealing at a specific temperature for each set of primers (see Table 1) for 20 s, extension at 72 °C for 30 s. Fluorescence data were acquired at 3 °C below the melting temperature of target genes to distinguish possible primer dimers. Products were verified by melting analysis and gel electrophoresis on 1.5% agarose gel with ethidium bromide staining.

Relative quantification analysis was performed using a dynamic amplification efficiency determination for each amplification run as provided in the comparative quantification function with the Rotor-Gene RG-3000 software (described in the technical bulletin entitled ''An Explanation of the Comparative Quantification Technique Used in the Rotor-Gene Analysis Software''; M. Herrmann, Corbett Research, Mortlake, Australia). The exact amplification efficiencies are assessed in each tube, and mathematic model is applied for the calculation of the normalized gene expression. The results for individual target genes were normalized according to the relative concentration of the external standard. Ratios of target gene concentration to luciferase mRNA concentration were estimated in each sample.

Table 1
Primers used for real time RT-PCR experiments

| Gene/transcript | Primers | Length (bp) | $T_{an}$ (°C) |
|---|---|---|---|
| Luciferase | F: 5′ – ACT TCG AAA TGT CCG TTC GG – 3′<br>R: 5′ – TCC GGA ATG ATT TGA TTG CC – 3′ | 633 | 55 |
| MATER | F: 5′ – GCT GGA GGC GTG TGG ACT G – 3′<br>R: 5′ – GGT CTG TAG ATT AGA GGT GGG ATG C – 3′ | 166 | 55 |
| H2AFZ | F: 5′– AGG ACG ACT AGC CAT GGA CGT GTG – 3′<br>R: 5′– CCA CCA CCA GCA ATT GTA GCC TTG – 3′ | 208 | 60 |
| HMGN2 (HMG-17)* | F: 5′ – GTA CAG TTT GAA ATA CTA TT – 3′<br>R: 5′ – GAA GTG TTC TGT GTG CT – 3′ | 110 | 47 |
| EIF4A2 (eIF-4A)* | F: 5′ – TTC TTT AAC ATT CAA ACT TC – 3′<br>R: 5′ – GCA TTT TGT TTG GTA TTA TA – 3′ | 166 | 50 |
| eIF4E | F: 5′ – GCA TCA TAC TCT TTC AAA CCA GC – 3′<br>R: 5′ – TGG CCC ACT CTG TAA TAG CTT – 3′ | 167 | 50 |
| CENPF (mitosin)* | F: 5′ – TTG TAA AGA AAG GGT TTG C – 3′<br>R: 5′ – CCA GCT GTT GGT TTG GAG G – 3′ | 172 | 50 |
| SFRS3 | F: 5′ – TGC TTA AGG GAA CAT TGT A – 3′<br>R: 5′ – ACA GAA AAT TCA AGT TTT G – 3′ | 182 | 50 |

MATER (Nlrp5, NLR family, pyrin domain containing 5); H2AFZ (H2A histone family, member Z); CENPF (centromere protein F, 350/400 ka); HMGN2 (high mobility group nucleosomal binding domain 2); HMG-17 (non-histone chromosomal protein HMG-17); SRFS3 (splicing factor, arginine/serine-rich 3); EIF4A2, eIF-4A, and EIF4E (eukaryotic translation initiation factors). $T_{an}$ = annealing temperature
* Previously known names in parentheses.

### 2.10. Statistical analysis

Normalization of the results obtained for individual genes within each pool of mRNA was performed by calculating each as a ratio to the level of luciferase mRNA. Data are presented in Figs. 1 and 2 as mean ± SD. The mean ± SD was obtained from three independent real-time RT-PCRs from three different batches of pooled embryos. The batches were collected from different oocyte fertilization/embryo cultivation experiments. Data on the relative concentration of templates were analyzed using the SigmaStat 3.0 (Jandel Scientific, San Rafael, CA, USA) software package. One-way ANOVA for repeated measures was used for the analysis of differences in the concentration of templates. P values $<0.05$ were considered significant.

## 3. Results

### 3.1. SMART cDNA synthesis

After SMART cDNA amplification, a smear of cDNA ranging from 300 bp to 1.2 kb in size was visualized by agarose gel electrophoresis in MII oocytes

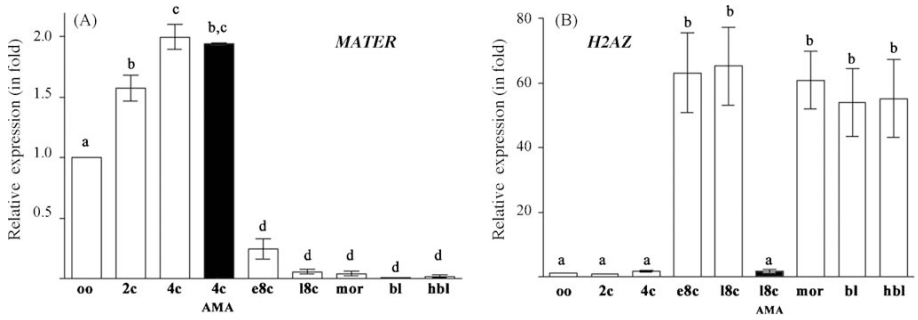


Fig. 1. Relative abundance of MATER and H2AFZ mRNAs during bovine preimplantation development. Poly(A)+ mRNA was extracted from pooled (20 in each group) oocytes and embryos (oo, MII oocyte; 2c, 2-cell; 4c, 4-cell; e8c, early 8-cell; l8c, late 8-cell stage; mor, morula; bl, blastocyst; hbl, hatched blastocyst). The luciferase mRNA as an external mRNA standard was added (1 pg/1 oocyte or embryo) before RNA isolation. The expression of selected genes was estimated by a one-step RT-PCR with real-time detection. The results for individual target genes were normalized according to the relative concentration of the external standard. The mean ± SD was obtained from three independent real-time RT-PCRs from three different batches of embryos. (A) MATER (Nlrp5, NLR family, pyrin domain containing 5); (B) H2AFZ (H2A histone family, member Z). The α-amanitin was added to the culture medium at a final concentration of 100 μg/mL at the time of minor genome activation (from late 1- to 4-cell stage). [a–d]Different superscripts above the columns indicate significant differences.

Table 2
Identity, percentage homology, and accession number of putative bovine 4-cell-stage embryo subtracted cDNA clones compared with known sequences in GenBank

| *Gene/transcript* | Homology (%) | Accession no. |
|---|---|---|
| *Bos taurus* similar to NACHT, LRR, and PYD containing protein 14 | 99 | AF 490528 |
| **Human HMG-17 gene for non-histone chromosomal protein HMG-17 (HMGN2)** | 83 | X13546.1 |
| *Bos taurus* isolate 65 NADH dehydrogenase subunits (ND1, ND2, ND3, ND4L, ND4, ND5, ND6), cytochrome oxidase subunits (COI, COII, COIII), ATPase 6, 8, and cytochrome b (CYTB) genes | 93 | AF 490528 |
| *Homo sapiens* bromodomain and PHD finger containing, 1, transcript variant 1 | 67 | BC053851 |
| PREDICTED: *Bos taurus* similar to leucine-rich repeats and calponin homology (CH) domain containing 3 | 99 | XM_870783.1 |
| ***Homo sapiens* eukaryotic translation initiation factor 4A, isoform 2 (EIF2A)** | 93 | BC015842 |
| PREDICTED: *Bos taurus* similar to jumonji domain containing 2D | 95 | XM_603100.2 |
| *Bos taurus* D-glucuronyl C5 epimerase | 99 | AF003927 |
| *Homo sapiens* 26S proteasome subunit 9 | 94 | AF001212.1 |
| *Bos taurus* mitochondrial RNA, similar to 12S | 94 | |
| *Bos taurus* poly(rC) binding protein 1 (PCBP1) | 99 | NM_001015565.1 |
| *Bos taurus* plasma membrane calcium-transporting ATPase | 95 | AF332982 |
| Human DNA sequence from clone RP11-409O11 on chromosome 9 contains the 5′ UTR of the TMEM2 gene for transmembrane protein 2, the 3′ end of a novel gene (CGI-67), and a CpG island | 80 | AL671309.6 |
| ***Bos taurus* eukaryotic translation initiation factor 4E (EIF4E)** | 99 | NM_174310 |
| *Mus musculus* partial mRNA for *N*-methyl-D-aspartate receptor 2B (Grin2b gene), splice variant 2 | 92 | AJ459262 |
| *Homo sapiens* nuclear receptor co-repressor 1 (NCOR1) | 88 | NM_006311.2 |
| *Homo sapiens* mitochondrial ribosomal protein L10, transcript variant 1 | 89 | BC052601.1 |
| PREDICTED: *Bos taurus* similar to Ssu72 RNA polymerase II CTD phosphatase homolog | 96 | XM_597314.2 |
| ***Homo sapiens* centromere protein F, 350/400ka (mitosin) (CENPF)** | 98 | NM 016343 |
| PREDICTED: *Pan troglodytes* similar to GMP reductase 2 (guanosine 5-monophosphate oxidoreductase 2) | 92 | XM_509866.1 |
| ***Homo sapiens* splicing factor, arginine/serine-rich 3 (SFRS3)** | 92 | NM_003017.3 |
| *Homo sapiens* ring finger protein 4 (RNF4) | 90 | NM_002938.2 |
| *Homo sapiens* mitochondrial ribosomal protein S7 (MRPS7) | 92 | NM_015971.2 |
| PREDICTED: *Bos taurus* similar to mannosidase, endo-alpha | 98 | XM_590447.2 |
| *Mus musculus* DNA polymerase N (Poln) | 97 | NM_181857.1 |
| *Homo sapiens* zinc finger protein 330 (ZNF330) | 75 | AJ006591.2 |
| *Bos taurus* thymosin beta 4 mRNA | 98 | AY192438.1 |
| Bovine gamma globin gene and globin (PSI-2) pseudogene | 86 | M63452.1 |
| *Homo sapiens* DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 (DDX39) | 88 | NM_005804.2 |
| PREDICTED: *Rattus norvegicus* oxidative-stress responsive 1 (predicted) (Osr1_predicted) | 82 | XM_236687 |
| Human DNA sequence from clone RP5-1183I21 on chromosome 20q12$^{-}$ Contains a novel gene, the 5′ end of a novel gene, the 5′ end of the JPH2 gene for junctophilin 2 (JP2, JP-2), and a CpG island | 91 | HS1183I21 |

*Note:* The expression of selected genes (shown in boldface) was estimated by a real-time RT-PCR detection.

and 4-cell-stage embryos. The optimum cycle number was then chosen as one cycle less than was needed to reach the plateau; that is, when product amplification was no longer observed on the gel (27 PCR cycles for MII oocyte samples, 25 PCR cycles for 4-cell-stage samples).

### 3.2. Suppressive subtractive hybridization

Following SSH and bacteria transfection, white colonies were picked and 200 inserts were PCR amplified. From these, 60 PCR products were selected for sequencing based on the length of the PCR products (more than 400 bases). The resulting partial sequences were compared with known sequences in the GenBank database, and the results are summarized in Table 2.

Of the 4-cell-stage embryos, 31 amplicons were homologous with identified gene product, thymosin beta 4 was identified in seven colony inserts, DNA polymerase N was identified in six colony inserts, and other amplicons were identified in one colony insert.

*3.3. Real-time quantitative RT-PCR*

We examined the expression of mRNAs encoding five different proteins with importance in cell division (centromere protein F, 350/400 ka [CENPF], previously known as mitosin), chromatin remodeling (high mobility group nucleosomal binding domain 2 [HMGN2], previously known as nonhistone protein HMG17), RNA splicing (SRFS3, previously known as splicing factor SRp20), and initiation of translation

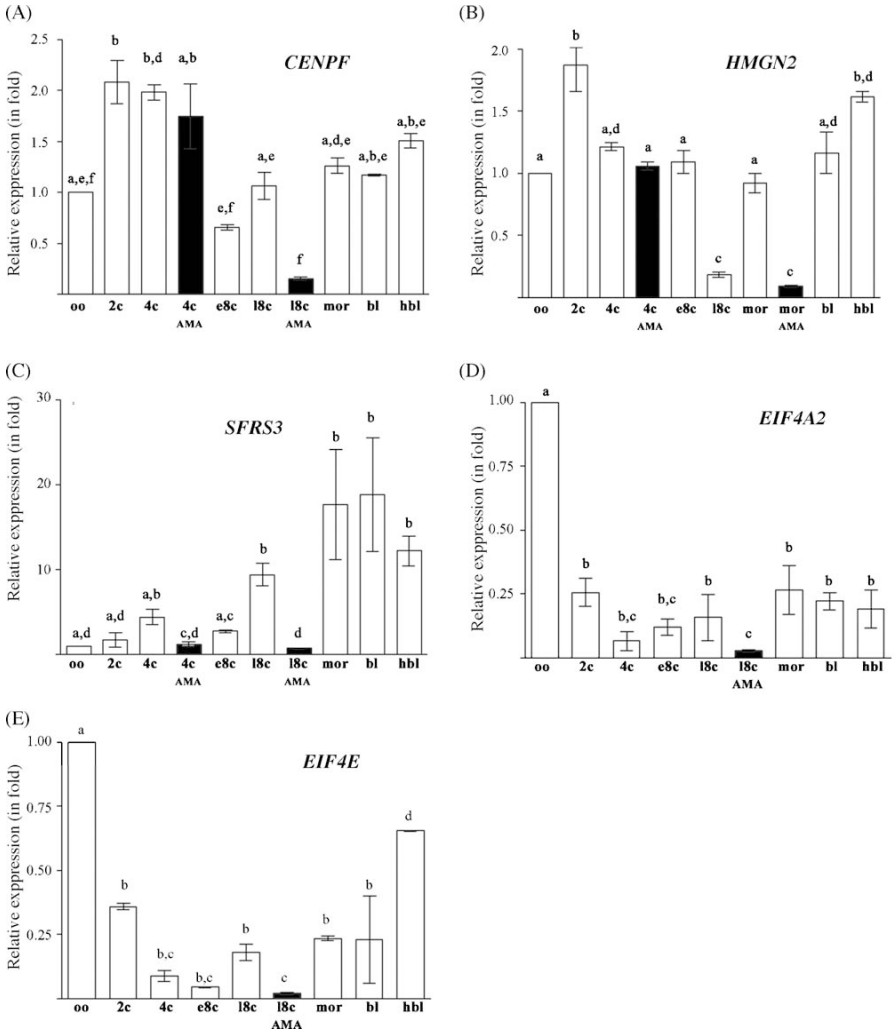


Fig. 2. Relative abundance of differentially expressed gene transcripts during bovine preimplantation development. Poly(A)+ mRNA was extracted from pooled (20 in each group) oocytes and embryos (oo, MII oocyte; 2c, 2-cell; 4c, 4-cell; e8c, early 8-cell; l8c, late 8-cell stage; mor, morula; bl, blastocyst; hbl, hatched blastocyst). The luciferase mRNA as an external mRNA standard was added (1 pg/1 oocyte or embryo) before RNA isolation. The expression of selected genes was estimated by a one-step RT-PCR with real-time detection. The results for individual target genes were normalized according to the relative concentration of the external standard. The mean ± SD was obtained from three independent real-time RT-PCRs from three different batches of embryos. (A) Centromere protein F, 350/400 ka (CENPF); (B) high mobility group nucleosomal binding domain 2 (HMGN2); (C) splicing factor, arginine/serine-rich 3 (SRFS3); (D) eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2); (E) eukaryotic translation initiation factor 4E (EIF4E). The α-amanitin was added to the culture medium at a final concentration of 100 μg/mL at the time of minor genome activation (from late 1- to 4-cell stage) and major genome activation (from 4- to late 8-cell stage; in the HMGN2 study from early 8-cell to morula stage). The embryos after α-amanitin treatment at the 4-cell stage, late 8-cell stage, and morula stage (in the HMGN2 study) were washed, frozen, and stored before RNA isolation and subsequent detection. In EIF4A2 and EIF4E experiments, the α-amanitin treatment was performed only during major genome activation. [a–f]Different superscripts above the columns indicate significant differences.

(eukaryotic translation initiation factors EIF4A2 and EIF4E). Moreover, maternal gene MATER (Nlrp5, NLR family, pyrin domain containing 5) as a negative control and H2AFZ (H2A histone family, member Z) gene as a positive control were included in our studies.

The expression of MATER mRNA gradually increased from oocyte until 4-cell stage. During further embryo development, its level rapidly dropped (Fig. 1A). H2AFZ mRNA was detectable in oocytes at a very low level. It considerably increased at early 8-cell stage; this increase was α-amanitin insensitive (Fig. 1B). The mRNA encoding CENPF was present in oocytes, its apparent abundance increased at the 2- to 4-cell stage, but this increase was α-amanitin insensitive (Fig. 2A). Another increase in mRNA content is visible at the late 8-cell stage; this increase is α-amanitin sensitive. We found a similar pattern of expression and α-amanitin sensitivity in mRNA encoding HMGN2 (Fig. 2B). In HMGN2, the increase in mRNA abundance is visible at morula stage, and this increase is α-amanitin sensitive. The mRNA encoding SRFS3 is present in very low amounts in oocytes; its expression increases slightly at the 4-cell stage, and this increase is α-amanitin sensitive (Fig. 2C). Another α-amanitin sensitive increase in its expression occurs at the late 8-cell stage, and the level of expression further increases until the blastocyst stage. Both eukaryotic translation initiation factors EIF4A2 (Fig. 2D) and EIF4E (Fig. 2E) exhibit similar patterns of mRNA expression. Their mRNAs are abundant in oocytes and decrease sharply until the 8-cell stage. Their mRNA level increases again in the late 8-cell stage, and this expression is α-amanitin sensitive.

## 4. Discussion

Real-time RT-PCR represents a convenient tool for the quantification of gene expression during mammalian embryonic development. However, the use of an internal standard often referred to as the housekeeping gene is essential for normalization of the mRNA levels. The finding of a suitable housekeeping gene is difficult due to the fact that the transcription of most genes changes rapidly during the early developmental period from oocyte to blastocyst. From eight tested genes, the authors selected H2A.1, a member of the H2A histone family, as the most acceptable housekeeping gene. The level of its transcription was constant during the bovine preimplantation period [37]. H2A.1 mRNA is largely of maternal origin and is found in both the polyadenylated and nonadenylated states. The priming strategy during reverse transcription has an important impact on H2A.1 mRNA level measurement. When the reverse transcription was primed with oligo-dT primers, this mRNA level was stable during the preimplantation development. In our experiments, we used one-tube real-time RT-PCR with specific set of primers, and for this reason oligo-dT primers are not suitable.

Vigneault et al. [38] recommended H2A.Z variant for interdevelopmental stage comparison during the pre-maternal zygotic transition period (MZT). However, it should be avoided for the standardization of developmental series spanning the pre- and post-MZT period (Fig. 1B). Because of the lack of a proper housekeeping gene, whose expression level is stable during the whole period of preimplantation development, we used luciferase mRNA as an external standard [39].

Maternal mRNAs are stored in an inactive, masked form and recruited for translation during oocyte maturation or early embryogenesis. Oocyte mRNAs containing short poly(A) tails are translationally inactive [18]. Their recruitment is accompanied by an elongation of the poly(A) tail at subsequent stages of development. This elongation between meiotic resumption and the first cleavage was monitored in bovine embryo. The obtained results indicate that mRNA polyadenylation changes in four different patterns, depending on the transcript examined [40]. It is evident that if the method of RNA isolation or reverse transcription was based on the poly(A) tail, this method could result in changes in mRNA abundance related to polyadenylation. Using one-step RT-PCR and specific primers in our experiments, our results of quantitative RT-PCR are not affected with the length of poly(A) tails. However, we used a sensitive method based on the binding of poly(A) tail on oligo d(T) particles (Dynabeads) for mRNA extraction. Extensive changes in the length of the poly(A) tail between the MII oocyte and 2- to 4-cell stage leads probably to observed differences in CENPF and HMGN2 mRNA abundances at these stages (Fig. 2), and our results are affected with these changes in the length of the poly(A) tail. This view is also supported by the fact that the same changes in mRNA abundance can be found in the maternal effect gene MATER, which serves as a control gene (Fig. 1A). The observed pattern of mRNA decrease during 4-cell stage to blastocyst corresponds approximately with the results published by Pennetier et al. [41]; the minimal differences can be caused by different cultivation media used in our experiments. It is not known whether extensive changes in the length of the poly(A) tail occur also between the 4-cell stage and blastocyst. Seeing that the short length of the poly(A) tail is also sufficient for

the effective isolation of mRNA through Dynabeads, it is unlikely that the pattern of mRNA abundance after 4-cell stage would be affected by the length of the poly(A) tail. Moreover, this method is generally used when transcription during early embryonic development is monitored. In addition, α-amanitin treatment serves as the control ensuring we are really monitoring newly synthesized mRNAs.

Suppression subtractive hybridization is a common method used for the discovery and isolation of genes that are differentially expressed between two cellular populations. Suppression subtractive hybridization has already been successfully used during preimplantation development of mammals for the discovery of gene expression during EGA [35,36,42] or the blastocyst stage [43]. Applying this method, we constructed a subtractive 4-cell-stage cDNA library using MII oocyte driver cDNA. Five different genes, which were discovered by SSH and which play an important role in the cell cycle, chromatin remodeling, splicing, or translation were further characterized by real-time quantitative RT-PCR. The α-amanitin treatment was used as evidence that the increase in RNA abundance really concerns newly synthesized mRNA and not just an extensive change in poly(A) tail length. Only with SRFS3 we proved that its new mRNA synthesis occurs at the 4-cell stage. The level of mRNAs of both CENPF and HMGN2 at the 4-cell stage does not decrease after cultivation with α-amanitin, therefore it does not concern newly synthesized mRNA. The levels of both EIF4E and EIF4A2 mRNAs are reduced significantly between the MII oocyte and 4-cell-stage embryo. It is obvious that there is no new mRNA synthesis; cultivation with α-amanitin was not performed for this reason. Therefore, only one of five observed genes repeats the expected expression pattern according to the SSH library. This SSH efficiency is low but it is comparable with the results obtained from SSH application on the early embryonic development of mammals. In an analysis of the expression of developmentally competent and incompetent bovine 2-cell-stage embryos using SSH, the authors subsequently tested the genes found using real-time RT-PCR. Only one of three observed genes repeated the pattern discovered by the SSH [35]. When the gene expression profiles of mouse zygote and oocyte are compared, only one of five genes tested by real-time RT-PCR repeats the expression pattern found by SSH [44]. These results could be supported by conclusion that abundant transcripts common to both tester and driver may escape both normalization and subtraction, giving rise to abundant background clones [42]. Nevertheless, the authors concluded that SSH can be considered as a tool of considerable potential when studying the onset of mammalian development.

The transcriptomes of bovine metaphase II oocytes and all stages of preimplantation embryos developing in vivo up to the blastocyst were recently determined using Affymetrix GeneChip [45]. Of the 23,000 transcripts examined, several hundred genes are transcribed before the 8-cell stage. The expression profile of six of those genes was checked by real-time RT-PCR with in vivo and in vitro embryos. The authors used Dynabeads for mRNA extraction, and there was no control cultivation with RNA inhibitors. Nevertheless, it is evident that a certain percentage of genes are transcribed even before major genome activation in the bovine embryos.

Pre-mRNA processing represents an important mechanism for modifying cellular protein composition during preimplantation development. It has been demonstrated that splicing factor p100 (formerly prp1/zer1/prp6) was gradually translocated into the pronuclei from cytoplasm in mouse 1-cell-stage embryos [46]. In our study, splicing factor SFRS3 mRNA was present during the preimplantation period; its expression increased at the level correlating with minor and major activation of gene expression. SFRS3 promotes the nucleocytoplasmic export of mRNAs in the mouse [47]. It can influence alternative polyadenylation [48]. The SFRS3 mRNA level is cell cycle regulated in mouse cells, and SFRS3 itself is alternatively spliced, apparently in a cell cycle–specific manner [49]. Mutant mouse embryos with inactivated SFRS3 gene failed to form a blastocyst and died at the morula stage [50]. In agreement with this result, we found SFRS3 mRNA expression during minor and major genome activation in 4-cell-stage and late 8-cell-stage bovine embryos. This expression is α-amanitin sensitive even during the minor genome activation period; after 24 h incubation with α-amanitin, SFRS3 mRNA synthesis is partly blocked. This result is in agreement with the role of SFRS3 in pre-mRNA processing during preimplantation development, including the onset of embryonic genome activation.

CENPF (mitosin) is a human 350-kDa kinetochore protein located at the outer kinetochore plate [51]. The protein level is low in the G1 phase and increases sharply in S and G2 phases; avian and murine CENPF proteins are expressed thorough the cell cycle [14,52]. Following the progression of the M phase, CENPF is hyperphosphorylated and exhibits colocalization at the kinetochores, spindle poles, and midbody [53]. Experimental depletion of CENPF results in pleiotropic mitotic defects including chromosome misalignment,

improper microtubule-kinetochore attachments, abnormal spindle morphology, and failure of chromosome segregation and cytokinesis [54,55]. Collectively, these observations suggest the role of CENPF in the cell cycle, M phase progression, and chromosome segregation. The expression of CENPF mRNA during major genome activation is in agreement with its important role in the mitotic cell cycle. To our knowledge, this is the first time CENPF mRNA expression was found during preimplantation development.

High mobility group (HMG) proteins are being recognized as essential factors in the regulation of nuclear functions. They distort or modify the structure of DNA complexes with transcription factors or histones [56,57]. HMGN2 is structurally unrelated to other HMG proteins; it constitutes the major family of chromosomal proteins and is present in chromatin at approximately 10% of the abundance of histones [58]. Chromatin containing transcribable genes is two- to threefold enriched in HMGN2 [59]; HMGN2 is concentrated in foci that colocalize with RNA polymerase II and nascent transcript. HMGN2 was identified as the constitutive component of mouse oocytes and embryonic chromatin [60]. Mouse embryos, from which HMGN2 had been depleted, manifested a reduced rate of RNA synthesis and developmental delay. In accordance with previous results, we found increases of HMGN2 mRNA content at 2- to 4-cell and morula stages bovine embryos; only the increase at morula stage is α-amanitin sensitive. We suggest that new HMGN2 mRNA synthesis is followed by synthesis of HMGN2 protein, which is necessary for modification of chromatin structure. The increase in HMGN2 mRNA expression correlates with major genome activation in bovine preimplantation development. HMGN2 probably plays a role in the modification of chromatin, which subsequently becomes transcriptionally permissive.

The transcripts of EIF4A2 and EIF4E found through SSH belong to eukaryotic translation initiation factors. The translational initiation complex EIF4F consists of eukaryotic initiation factor 4E (EIF4E) in a stable complex with two other molecules, EIF4A2 (RNA helicase) and EIF4G1, a putative mRNA-ribosome ‘‘bridging factor’’ [61]. EIF4E is the control component in the initiation and regulation of translation in the eukaryotic cell; it promotes the transport of mRNAs of a specific set of transcripts, such as cyclin D1, but not of housekeeping genes [62]. Consistent with these roles, EIF4E is required for cell cycle progression, it exhibits antiapoptotic activity, and, when overexpressed, transforms cells [61]. Both eukaryotic initiation factors (eIF4E and EIF4A2), whose mRNA we found during bovine preimplantation development, show the same pattern of mRNA abundance (i.e., the increase in their mRNA abundance correlates with the major genome activation period).

The increase in mRNA content of all other examined genes at the late 8-cell stage, eventually later, can be inhibited with α-amanitin; therefore it is demonstrably a matter of the synthesis of new embryonic mRNA during major genome activation. HMGN2-depleted mouse embryos showed a reduced rate of RNA synthesis and a delay in embryonic development. CENPF-depleted cells showed M-phase delay, premature chromosome decondensation, and massive cell death. The studied genes can therefore play an important role in preimplantation development. A functional study using RNA interference experiments during preimplantation bovine embryo development is in progress.

The results of this study represent a quantification of mRNA abundance of five different genes, which we found by means of SSH and which play an important role in bovine preimplantation development. Only SFRS3 transcription in the 4-cell stage is significantly inhibited by α-amanitin. SRFS3 represents a gene with apparent function, whose new synthesis is directed by the bovine embryonic genome during minor genome activation. It is already known that mouse embryos with inactivated SFRS3 gene died at the morula stage.

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## References

[1] Schultz RM, Davis W, Stein P, Svoboda P. Reprogramming of gene expression during preimplantation development. J Exp Zool 1999;285:276–82.
[2] Latham KE, Schultz RM. Embryonic genome activation. Front Biosci 2001;6:D748–759.
[3] Kanka J. Gene expression and chromatin structure in the preimplantation embryo. Theriogenology 2003;59:3–19.
[4] Bettegowda A, Lee KB, Smith GW. Cytoplasmic and nuclear determinants of the maternal-to-embryonic transition. Reprod Fertil Dev 2008;20:45–53.
[5] Clegg KB, Piko L. Poly(A) length, cytoplasmic adenylation and synthesis of poly(A)+ RNA in early mouse embryos. Dev Biol 1983;95:331–41.
[6] Bensaude O, Babinet C, Morange M, Jacob F. Heat shock proteins, first major products of zygotic gene activity in mouse embryo. Nature 1983;305:331–3.

[7] Conover JC, Temeles GL, Zimmermann JW, Burke B, Schultz RM. Stage-specific expression of a family of proteins that are major products of zygotic gene activation in the mouse embryo. Dev Biol 1991;144:392–404.
[8] Latham KE, Solter D, Schultz RM. Activation of a 2-cell stage-specific gene following transfer of heterologous nuclei into enucleated mouse embryos. Mol Reprod Dev 1991;30: 182–6.
[9] Bouniol C, Nguyen E, Debey P. Endogenous transcription occurs at the 1-cell stage in the mouse embryo. Exp Cell Res 1995;218:57–62.
[10] Aoki F, Worrad DM, Schultz RM. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev Biol 1997;181:296–307.
[11] Christians E, Campion E, Thompson EM, Renard JP. Expression of the HSP 70.1 gene, a landmark of early zygotic activity in the mouse embryo, is restricted to the first burst of transcription. Development 1995;121:113–22.
[12] Zeng F, Schultz RM. RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. Dev Biol 2005;283:40–57.
[13] Svoboda P, Stein P, Anger M, Bernstein E, Hannon GJ, Schultz RM. RNAi and expression of retrotransposons MuERV-L and IAP in preimplantation mouse embryos. Dev Biol 2004;269: 276–85.
[14] Ma L, Zhao X, Zhu X. Mitosin/CENP-F in mitosis, transcriptional control, and differentiation. J Biomed Sci 2006;13: 205–13.
[15] Zeng FY, Baldwin DA, Schultz RM. Transcript profiling during preimplantation mouse development. Dev Biol 2004;272: 483–96.
[16] Hamatani T, Carter MG, Sharov AA, Ko MS. Dynamics of global gene expression changes during mouse preimplantation development. Dev Cell 2004;6:117–31.
[17] Wang QT, Piotrowska K, Ciemerych MA, Milenkovic L, Scott MP, Davis RW, Zernicka-Goetz M. A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. Dev Cell 2004;6:133–44.
[18] Potireddy S, Vassena R, Patel BG, Latham KE. Analysis of polysomal mRNA populations of mouse oocytes and zygotes: dynamic changes in maternal mRNA utilization and function. Dev Biol 2006;298:155–66.
[19] Hara KT, Oda S, Naito K, Nagata M, Schultz RM, Aoki F. Cyclin A2-CDK2 regulates embryonic gene activation in 1-cell mouse embryos. Dev Biol 2005;286:102–13.
[20] Philipsen S, Suske G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res 1999;27:2991–3000.
[21] Bultman SJ, Gebuhr TC, Pan H, Svoboda P, Schultz RM, Magnuson T. Maternal BRG1 regulates zygotic genome activation in the mouse. Genes Dev 2006;20:1744–54.
[22] Camous S, Kopecny V, Flechon JE. Autoradiographic detection of the earliest stage of [3/H]-uridine incorporation into the cow embryo. Biol Cell 1986;58:195–200.
[23] Frei RE, Schultz GA, Church RB. Qualitative and quantitative changes in protein synthesis occur at the 8-16-cell stage of embryogenesis in the cow. J Reprod Fertil 1989;86: 637–41.
[24] DeLouis C, Bonnerot C, Vernet M, Nicolas JF. Expression of microinjected DNA and RNA in early rabbit embryos: changes in permissiveness for expression and transcriptional selectivity. Exp Cell Res 1992;201:284–91.
[25] Viuff D, Avery B, Greve T, King WA, Hyttel P. Transcriptional activity in in vitro produced bovine two- and four-cell embryos. Mol Reprod Dev 1996;43:171–9.
[26] Memili E, Dominko T, First NL. Onset of transcription in bovine oocytes and preimplantation embryos. Mol Reprod Dev 1998;51:36–41.
[27] Memili E, First NL. Control of gene expression at the onset of bovine embryonic development. Biol Reprod 1999;61:1198–207.
[28] Liu ZS, Foote RH. Effects of amino acids and alpha-amanitin on bovine embryo development in a simple protein-free medium. Mol Reprod Dev 1997;46:278–85.
[29] Natale DR, Kidder GM, Westhusin ME, Watson AJ. Assessment by differential display-RT-PCR of mRNA transcript transitions and alpha-amanitin sensitivity during bovine preattachment development. Mol Reprod Dev 2000;55:152–63.
[30] Kanka J, Bryova A, Duranthon V, Oudin JF, Peynot N, Renard JP. Identification of differentially expressed mRNAs in bovine preimplantation embryos. Zygote 2003;11:43–52.
[31] Jakobsen AS, Avery B, Dieleman SJ, Knijn HM, Vos PL, Thomsen PD. Transcription of ribosomal RNA genes is initiated in the third cell cycle of bovine embryos. Mol Reprod Dev 2006;73:196–205.
[32] Misirlioglu M, Page GP, Sagirkaya H, Kaya A, Parrish JJ, First NL, Memili E. Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. Proc Natl Acad Sci USA 2006;103:18905–10.
[33] Vigneault C, Gravel C, Vallee M, McGraw S, Sirard MA. Unveiling the bovine embryo transcriptome during the maternal-to-embryonic transition. Reproduction 2009;137:245–57.
[34] Pavlok A, Lucas-Hahn A, Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev 1992;31:63–7.
[35] Fair T, Murphy M, Rizos D, Moss C, Martin F, Boland MP, Lonergan P. Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. Mol Reprod Dev 2004;67:136–44.
[36] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[37] Robert C, McGraw S, Massicotte L, Pravetoni M, Gandolfi F, Sirard MA. Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. Biol Reprod 2002;67:1465–72.
[38] Vigneault C, Gilbert I, Sirard MA, Robert C. Using the histone H2a transcript as an endogenous standard to study relative transcript abundance during bovine early development. Mol Reprod Dev 2007;74:703–15.
[39] Brunet-Simon A, Henrion G, Renard JP, Duranthon V. Onset of zygotic transcription and maternal transcript legacy in the rabbit embryo. Mol Reprod Dev 2001;58:127–36.
[40] Brevini TAL, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. Mol Reprod Dev 2002;63:510–7.
[41] Pennetier S, Perreau C, Uzbekova S, Thelie A, Delaleu B, Mermillod P, Dalbies-Tran R. MATER protein expression and intracellular localization throughout folliculogenesis and preimplantation embryo development in the bovine. BMC Dev Biol 2006;6:26.
[42] Bui LC, Leandri RD, Renard JP, Duranthon V. SSH adequacy to preimplantation mammalian development: scarce specific tran-

scripts cloning despite irregular normalisation. BMC Genomics 2005;6:155.

[43] Mohan M, Ryder S, Claypoo PL, Geisert RD, Malayer JR. Analysis of gene expression in the bovine blastocyst produced in vitro using suppression-subtractive hybridization. Biol Reprod 2002;67:447–53.

[44] Yao YQ, Xu JS, Lee WM, Yeung WS, Lee KF. Identification of mRNAs that are up-regulated after fertilization in the murine zygote by suppression subtractive hybridization. Biochem Biophys Res Commun 2003;304:60–6.

[45] Kues WA, Sudheer S, Herrmann D, Carnwath JW, Havlicek V, Besenfelder U, et al. Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo. Proc Natl Acad Sci USA 2008;105:19768–73.

[46] Nishikimi A, Mukai J, Ikeda S, Yamada M. Nuclear translocation of a pre-mRNA splicing factor, p100(prp1/zer1/prp6), in mouse 1-cell embryos. J Reprod Dev 2002;48:257–63.

[47] Huang Y, Steitz JA. Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. Mol Cell 2001;7:899–905.

[48] Lou H, Neugebauer KM, Gagel RF, Berget SM. Regulation of alternative polyadenylation by u1 snRNPs and SRp20. Mol Cell Biol 1998;18:4977–85.

[49] Jumaa H, Guenet JL, Nielsen PJ. Regulated expression and RNA processing of transcripts from the Srp20 splicing factor gene during the cell cycle. Mol Cell Biol 1997;17:3116–24.

[50] Jumaa H, Wei G, Nielsen PJ. Blastocyst formation is blocked in mouse embryos lacking the splicing factor SRp20. Curr Biol 1999;9:899–902.

[51] Liao H, Winkfein RJ, Mack G, Rattner JB, Yen TJ. CENP-F is protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. J Cell Biol 1995;130:507–18.

[52] Evans HJ, Edwards L, Goodwin RL. Conserved C-terminal domains of mCenp-F (LEK1) regulate subcellular localization and mitotic checkpoint delay. Exp Cell Res 2007;313: 2427–37.

[53] Liu ST, Hittle JC, Jablonski SA, Campbell MS, Yoda K, Yen TJ. Human CENP-I specifies localization of CENP-F, MAD1 and MAD2 to kinetochores and is essential for mitosis. Nat Cell Biol 2003;5:341–5.

[54] Holt SV, Vergnolle MAS, Hussein D, Wozniak MJ, Allan VJ, Taylor SS. Silencing Cenp-F weakens centromeric cohesion, prevents chromosome alignment and activates the spindle checkpoint. J Cell Sci 2005;118:4889–900.

[55] Varis A, Salmela AL, Kallio MJ. Cenp-F (mitosin) is more than a mitotic marker. Chromosoma 2006;115:288–95.

[56] Bustin M. Chromatin unfolding and activation by HMGN chromosomal proteins. Trends Biochem Sci 2001;26:431–7.

[57] Agresti A, Bianchi ME. HMGB proteins and gene expression. Curr Opin Genet Dev 2003;13:170–8.

[58] Bustin M, Reeves R. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Progr Nucleic Acid Res 1996;54:35–100.

[59] Postnikov YV, Shick VV, Belyavsky AV, Khrapko KR, Brodolin KL, Nikolskaya TA, Mirzabekov AD. Distribution of high mobility group preoteins 1/2, E and 14/17 and linker histone-H1 and histone-H5 on transcribed and nontranscribed regions of chicken erythrocyte chromatin. Nucleic Acids Res 1991;19: 717–25.

[60] Mohamed OA, Bustin M, Clarke HJ. High-mobility group proteins 14 and 17 maintain the timing of early embryonic development in the mouse. Dev Biol 2001; 229:237–49.

[61] Raught B, Gingras AC. eIF4E activity is regulated at multiple levels. Int J Biochem Cell Biol 1999;31:43–57.

[62] Topisirovic I, Capili AD, Borden KLB. Gamma interferon and cadmium treatments modulate eukaryotic initiation factor 4E-dependent mRNA transport of cyclin D1 in a PML-dependent manner. Mol Cell Biol 2002;22:6183–6198.

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# Silencing *CENPF* in bovine preimplantation embryo induces arrest at 8-cell stage

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## Abstract

**Identification of genes that are important for normal preimplantation development is essential for understanding the basics of early mammalian embryogenesis. In our previous study, we have shown that *CENPF* (mitosin) is differentially expressed during preimplantation development of bovine embryos. CENPF is a centromere–kinetochore complex protein that plays a crucial role in the cell division of somatic cells. To our best knowledge, no study has yet been done on either bovine model, or oocytes and preimplantation embryos. In this study, we focused on the fate of bovine embryos after injection of *CENPF* double-stranded RNA (dsRNA) into the zygotes. An average decrease of *CENPF* mRNA abundance by 94.9% or more and an extensive decline in immunofluorescence staining intensity was detected relative to controls. There was no disparity between individual groups in the developmental competence before the 8-cell stage. However, the developmental competence rapidly decreased then and only 28.1% of *CENPF* dsRNA injected 8-cell embryos were able to develop further (uninjected control: 71.8%; green fluorescent protein dsRNA injected control: 72.0%). In conclusion, these results show that depletion of *CENPF* mRNA in preimplantation bovine embryos leads to dramatic decrease of developmental competence after embryonic genome activation.**

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## Introduction

Identification of genes that are important for normal preimplantation development of mammals is essential for studying early mammalian embryogenesis. A large number of genes expressed from the embryonic genome during embryonic genome activation (EGA) were identified using all sorts of molecular genetic methods including microarray analysis (Hamatani *et al*. 2004, Wang *et al*. 2004, Misirlioglu *et al*. 2006, Kanka *et al*. 2009, Vigneault *et al*. 2009). However, the functions of many transcripts during mammalian embryogenesis are still not known. This study concerns one of these genes encoding *CENPF* (centromeric protein F; mitosin).

CENPF is a large human protein (>350 kDa; 3113 amino acids), which plays a crucial role in cell division by controlling microtubule dynamics, maintaining chromosome condensation, transcription regulation, and cell cycle progression (Liao *et al*. 1995, Zhu *et al*. 1995, Holt *et al*. 2005, Zhou *et al*. 2005). It is expressed and localized in a cell-cycle-dependent manner (Liao *et al*. 1995, Zhu *et al*. 1995). The protein starts to be expressed in the G1/S phase when it is dispersed in nucleoplasm with the exception of nucleolus (Zhu *et al*. 1995). During late G2 phase, it also relocalizes to the inner site of nuclear membrane and consequently to the outer plate of the forming prekinetochores (Liao *et al*. 1995, Zhu *et al*. 1995). CENPF is one of the earliest proteins associated with kinetochores (Bomont *et al*. 2005, Yang *et al*. 2005, Pouwels *et al*. 2007) and helps to form the correct kinetochore–microtubule interactions (Yang *et al*. 2005). The protein remains associated with kinetochores until chromosome segregation when it subsequently relocalizes to the spindle mid-zone and intracellular bridge (Liao *et al*. 1995, Zhu *et al*. 1995). CENPF reaches its maximum level at the G2/M transition and is rapidly degraded after the cell division (Zhu *et al*. 1995). Several studies have shown that the depletion of CENPF in somatic cells prevents correct chromosome alignment, destabilizes the microtubule–kinetochore interaction and weakens the tension between sister centromeres (Bomont *et al*. 2005, Holt *et al*. 2005, Yang *et al*. 2005).

The cell cycle during mammalian preimplantation development is very specific in many ways. The cycle is markedly shortened – especially the G1 phase – and at the same time, mitosis occupies longer part of the cycle (Bolton *et al*. 1984, Iwamori *et al*. 2002). The transcription of embryonic genome starts in bovines in the late 8-cell stage (8c; Camous *et al*. 1986, King *et al*. 1988, Kopecny *et al*. 1989, Pavlok *et al*. 1993) and this event is called EGA. Until EGA, all the mRNAs


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and proteins are of maternal origin (Bilodeau-Goeseels & Schultz 1997). Some authors suggest that there is also a so-called minor genome activation between 1- and 4c, which is followed by major genome activation in the 8c (Memili & First 2000, Jakobsen *et al.* 2006). We have recently proved that in bovine embryos the transcription of embryonic *CENPF* starts during major genome activation at late 8c. Until then, all the *CENPF* mRNA is of maternal origin and its amount gradually decreases from 2- to early 8c. After EGA, the expression level increases again and remains almost the same up until the blastocyst stage (Kanka *et al.* 2009).

Even though the embryo develops without any need of exogenous mitogens, it is very sensitive to changes of external environment. This is the cause of decreased developmental competence of *in vitro* produced embryos compared to embryos produced *in vivo* and a significant negative impact on the offspring (Hales & Barker 2001, DeBaun *et al.* 2003, Ecker *et al.* 2004).

In this study, we used the *CENPF*-specific double-stranded RNA (dsRNA) to silence the corresponding mRNA, so that we could monitor the developmental competence of the embryos and consequently compare the role of CENPF in mammalian preimplantation development with somatic cells.

## Results

### *Effect of* CENPF *dsRNA injection on embryonic* CENPF *mRNA expression*

To confirm that *CENPF* expression is needful for correct preimplantation development, we employed the RNA interference (RNAi) method. The dsRNA used was homologous to nucleotides 8971–9383 at the 3′ end of bovine *CENPF* mRNA. The microinjection of *CENPF* dsRNA efficiently and specifically causes degradation of *CENPF* mRNA in bovine preimplantation embryos (Fig. 1).

At late 8c, the *CENPF* mRNA was reduced by 96.0% ($P<0.001$) in comparison to uninjected control and by 94.9% ($P<0.001$) in comparison to green fluorescent protein (*GFP*) dsRNA injected control (Fig. 1A). At late 16-cell stage (16c), the *CENPF* mRNA was reduced by 97.8% ($P<0.02$) in comparison to uninjected control and by 98.5% ($P<0.002$) in comparison to *GFP* dsRNA injected control (Fig. 1B). No significant difference was found in the abundance of *CENPF* mRNA between the uninjected group and the *GFP* dsRNA injected group ($P>0.05$).

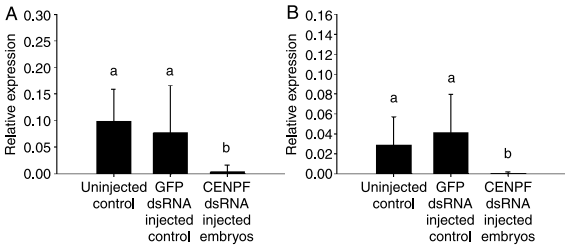


**Figure 1** Relative abundance of *CENPF* mRNA after injection of *CENPF* dsRNA. *CENPF* mRNA expression (A) in 8-cell stage embryos and (B) in 16-cell stage embryos. The relative abundance (*y*-axis) represents the amount of *CENPF* mRNA in a single embryo normalized to one blastomere. Bars show mean±S.D. [a,b]Values with different superscripts indicate statistical significance ($P<0.05$).

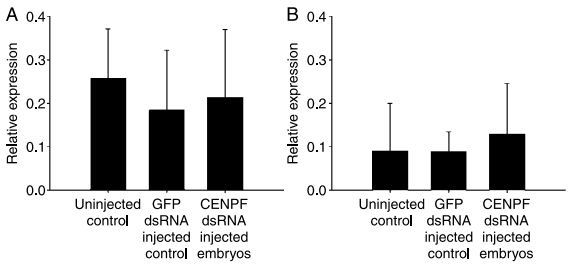


**Figure 2** The expression of control genes after injection of *CENPF* dsRNA. The relative abundance of (A) *NPM1* mRNA and (B) *H2AFZ* mRNA. The relative abundance (*y*-axis) represents the amount of mRNA in a single embryo normalized to one blastomere. Bars show mean±S.D.

To verify the specificity of *CENPF* mRNA degradation, we measured the level of mRNA of two control genes: H2A histone family, member Z (*H2AFZ*) and nucleophosmin (*NPM1*). No significant distinction between individual groups was detected ($P>0.05$ in each case; Fig. 2).

### *Effect of* CENPF *dsRNA injection on protein expression*

To monitor the effect of *CENPF* mRNA silencing on protein expression, we performed the immunofluorescence analysis using the polyclonal anti-CENPF antibody specific for C-terminus of the protein (Fig. 3). In uninjected embryos and embryos injected with *GFP* dsRNA, CENPF clearly colocalizes with the nuclei of blastomeres (Fig. 3A and B). In *CENPF* dsRNA injected embryos, we did not detect a similar localization pattern and fluorescence intensity was dramatically decreased (Fig. 3C).

### *Effect of* CENPF *mRNA silencing on developmental competence of the embryo*

We monitored the number of embryos arrested at individual developmental stages in each treatment group. No developmental impairment was noticed until EGA (8c; $P>0.05$ in each case; Fig. 4). However, a significantly lower number of *CENPF* dsRNA injected 8-cell embryos (8c) was capable to develop to 16c or beyond when compared with control groups (mean ±S.E.M.: uninjected control: 71.8%±3.55; *GFP* dsRNA injected control: 72.0%±2.62; *CENPF* dsRNA injected group: 28.1%±6.19; $P<0.001$ in both cases; Fig. 5). Moreover, the embryos in both control groups were of

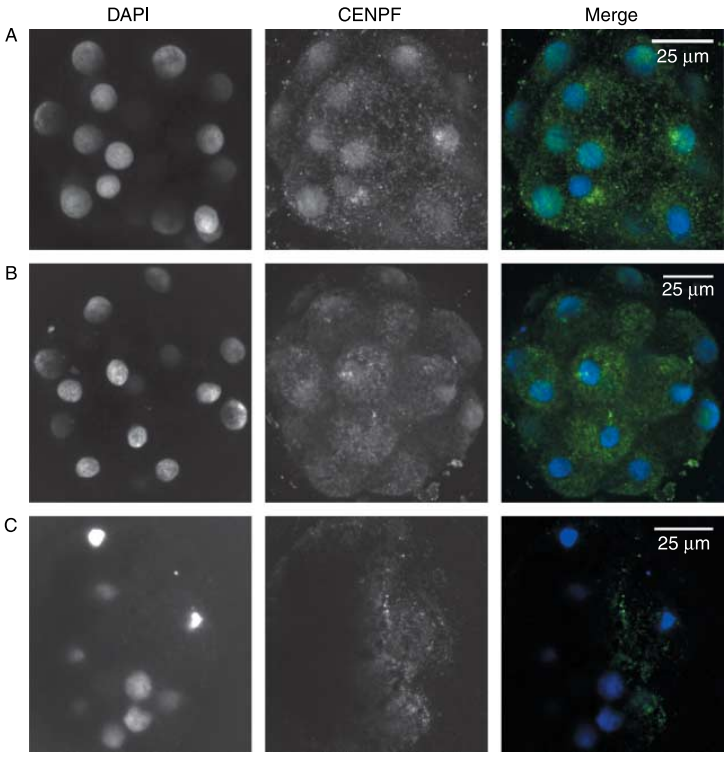


**Figure 3** The immunofluorescence detection of CENPF after injection of *CENPF* dsRNA. CENPF expression (A) in uninjected embryos; (B) in embryos injected with *GFP* dsRNA; (C) in embryos injected with *CENPF* dsRNA. The embryos were stained using specific anti-CENPF antibody against C-terminus (CENPF, green; DNA, blue). The embryos were fixed at 4.5 days post fertilization.

higher morphological quality. The most frequent defect in *CENPF* dsRNA injected embryos was an unequal size of blastomeres, indistinct boundaries of blastomeres and partial transparency of blastomeres. Immunofluorescence analysis revealed that some of the *CENPF* dsRNA injected embryos had fragmented nuclei or even the blastomeres did not have any nucleus (Fig. 3C). Only 33.02%±3.684 (mean±S.E.M.) of *CENPF* dsRNA injected embryos corresponded to the appropriate phenotype, whilst 68.88%±6.26 of uninjected embryos and 69.38%±10.38 *GFP* dsRNA injected embryos were of high quality ($P<0.05$ in both cases).

### *CENPF is not degraded before EGA in bovine preimplantation embryos*

To find out whether CENPF is cyclically degraded and resynthesized in pre-EGA embryos, we blocked the protein synthesis using translation inhibitor cycloheximide (CHX) during cultivation from late 4c to 8c embryos (4c–8c group) and from late 8c to 16c (8c–16c group). The embryos were then examined for CENPF presence using immunofluorescence analysis. No significant difference in staining intensity was found between CHX-treated and non-treated embryos in 4c–8c group (Fig. 6A and B). This suggests that CENPF is not degraded at the end of cell cycle in bovine preimplantation embryos before EGA. However, the results were considerably different in 8c–16c group. We did not observe complete degradation of CENPF, though, but the staining intensity was markedly weaker and the protein was not present in all the nuclei (Fig. 6C and D).

### *The localization of CENPF in bovine preimplantation embryos after EGA is cell cycle dependent*

We employed immunofluorescence analysis for the monitoring of CENPF localization during early embryo cell cycle. Our data suggest that bovine CENPF is expressed and localized in the same pattern as the human protein. Most of the blastomeres of the analysed embryos were in interphase. In consistence with the immunofluorescence staining in somatic cells (Liao *et al.* 1995, Zhu *et al.* 1995, Hussein & Taylor 2002, Feng *et al.* 2006), CENPF was detected in the whole nucleus except nucleoli (Fig. 7A and C; non-marked blastomeres). As the chromosomes gradually condense, fluorescent dots begin to appear on chromosomes (Fig. 7A; blastomere marked by arrowhead and B), which is consistent with the kinetochore localization during prophase and prometaphase in somatic cells

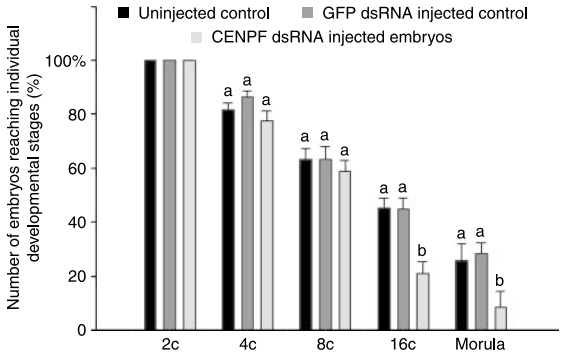


**Figure 4** Developmental competence of embryos after injection of *CENPF* dsRNA. Number of embryos reaching individual developmental stages (*y*-axis). The number of 2-cell stage embryo is considered as 100%. The development competence was followed up during 12 independent experiments. [a,b]Values with different superscripts indicate statistical significance ($P<0.05$). 2c, 2-cell stage embryos, 4c, 4-cell stage embryos, 8c, 8-cell stage embryos, 16c, 16-cell stage embryos.

(Liao *et al.* 1995, Zhu *et al.* 1995, Hussein & Taylor 2002, Feng *et al.* 2006). At the end of mitosis, CENPF ceases to be detectable by immunofluorescence (Fig. 7C; blastomere marked by arrowhead), which corresponds to the degradation of CENPF in somatic cells (Liao *et al.* 1995, Zhu *et al.* 1995, Hussein & Taylor 2002, Feng *et al.* 2006). The cell cycle stage of the blastomere was determined by DAPI staining.

## Discussion

Although CENPF is known to be crucially important for cell division and cell cycle progression, no study concerning early mammalian embryo development has been done, up to now. We have recently shown that *CENPF* mRNA expression from embryonic genome is activated at late 8c (Kanka *et al.* 2009), which suggest importance of *CENPF* expression during preimplantation development. To confirm this, we included CENPF in a more thorough study. The bovine model has been chosen

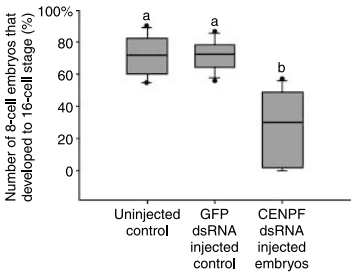


**Figure 5** Developmental competence of 8-cell embryos. Number of 8 cell stage embryos that developed to 16-cell stage or beyond (*y*-axis). The graph plots the median and 10th, 25th, 75th and 90th percentiles and outliers (dots). [a,b]Values with different superscripts indicate statistical significance ($P<0.05$).

because of the similarity of human and bovine preimplantation development (Telford *et al.* 1990, Adjaye *et al.* 2007, Kues *et al.* 2008).

The RNAi using dsRNA was used to silence the *CENPF* expression. The RNAi causes specific degradation of mRNA and is in fact the only applicable method for studying gene function in early mammalian embryo. Since the quality of early mammalian embryo unwinds from the quality of oocyte and the internal maternal environment, it is not feasible to use knockout for our purpose. For the assessment of degradation efficiency in single embryos we employed the FastLane Cell SYBR Green Kit (Qiagen) and the procedure that was first used by P Šolc for analysing single oocytes (personal communication; details described in Materials and Methods).

The absolute majority of studies concerning CENPF have been done on human somatic cells. Only four of its orthologs – murine CENPF (Goodwin *et al.* 1999, Ashe *et al.* 2003, Dees *et al.* 2005, Soukolis *et al.* 2005, Evans *et al.* 2007), avian CMF1 (Wei *et al.* 1996, Redkar *et al.* 2002) and worm hcp1 and hcp2 (Cheeseman *et al.* 2005, Hajeri *et al.* 2008) have been studied slightly more intensively. However, these proteins are expressed throughout the whole cell cycle, do not strictly localize to the nucleus or have a somewhat different function (Wei *et al.* 1996, Goodwin *et al.* 1999, Redkar *et al.* 2002, Cheeseman *et al.* 2005, Dees *et al.* 2005, Soukolis *et al.* 2005, Evans *et al.* 2007, Hajeri *et al.* 2008). This suggests that bovine CENPF does not necessarily have to be expressed in a cell-cycle-dependent manner. Moreover, some of the physiological activators and inhibitors of somatic cell cycle do not play the same role during preimplantation development and may even not be needed (Iwamori *et al.* 2002).

In somatic cells, CENPF participates in the kinetochore–microtubule interaction and is required for chromosome condensation, alignment and segregation. The silencing of CENPF in human somatic cells causes weakened centromere cohesion, premature chromosome decondensation and aneuploidy or metaphase arrest (Bomont *et al.* 2005, Holt *et al.* 2005, Yang *et al.* 2005).

The immunofluorescence analysis of bovine preimplantation embryo at the 16c exhibits the same expression and localization of CENPF as human somatic cells (Liao *et al.* 1995, Zhu *et al.* 1995, Hussein & Taylor 2002, Feng *et al.* 2006). In non-mitotic blastomeres we detected CENPF dispersed in nucleoplasm; in prophase and prometaphase blastomeres distinct foci of CENPF staining were detected on chromosomes. The protein was not detectable at the end of mitosis in preimplantation embryos after EGA (Fig. 7C). In somatic cells CENPF is degraded at the end of mitosis (Zhu *et al.* 1995). However, in preimplantation embryos before EGA, we did not detect any observable fall of protein amount after 24 h treatment with translation inhibitor CHX, which

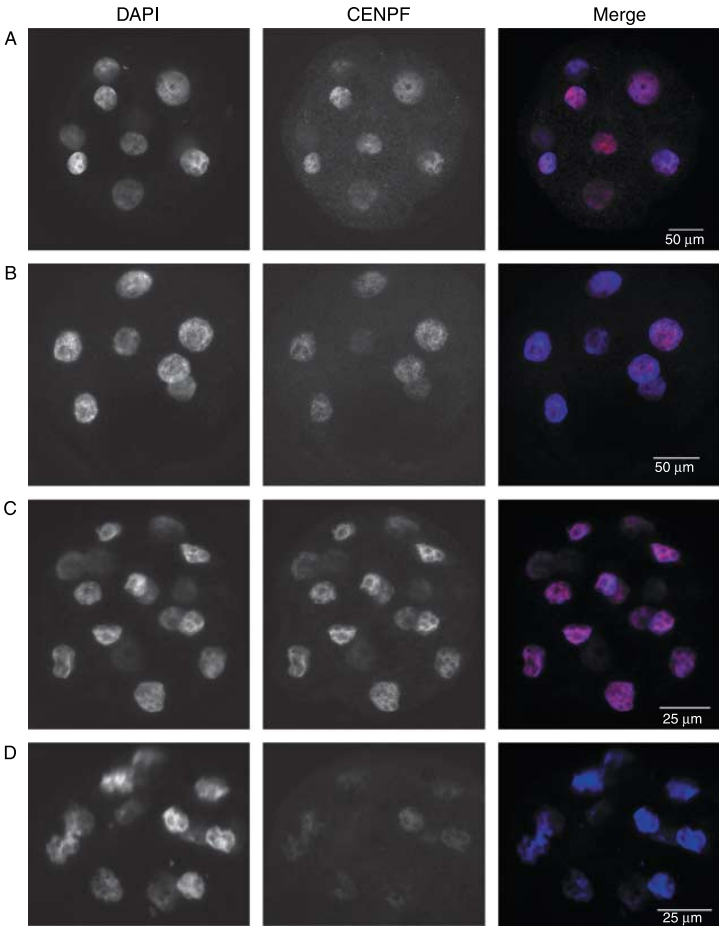


**Figure 6** CENPF expression in cycloheximide treated embryos. Immunofluorescence analysis of (A) non-treated 8-cell embryos, (B) embryos cultivated from late 4-cell stage to 8-cell stage (for 24 h) in 10 μg/ml cycloheximide, (C) non-treated 16-cell embryos, (D) embryos cultivated from late 8-cell stage to 16-cell stage (for 24 h) in 10 μg/ml cycloheximide. The embryos were stained using specific mouse anti-CENPF antibody against N-terminus (DAPI, blue; CENPF, red).

poses a sufficiently long period for passing through the cell cycle (Fig. 6A and B). On the other hand, after CHX treatment of embryos post EGA, we detected a considerable decline of staining intensity (Fig. 6C and D). Hence, we suppose the preservation of maternal CENPF protein until the EGA in the late 8c. On the basis of the data mentioned above, we supposed that the *CENPF*-depleted embryos might arrest after EGA.

To confirm this, we performed the dsRNA mediated specific degradation of *CENPF* mRNA. We did not notice any differences in developmental competence between individual treatment groups until the 8c ($P > 0.05$ in each case; Fig. 4). However, the developmental competence of *CENPF* dsRNA injected embryos steeply decreased after the 8c; lesser than one-third of 8c embryos reached the 16c (Fig. 5) and only rare embryo-survivors further developed. Similarly, POU5F1 (Oct-4)-depleted embryos are able to develop until the morula stage without any significant differences in developmental competence, although the maternal mRNA can be detected in embryos before EGA and the first embryonic transcript in bovine can be detected at the morula stage (Nganvongpanit *et al.* 2006*a*, 2006*b*). In our previous study (Kanka *et al.* 2009), we have shown that *CENPF* transcription from embryonic genome is activated during major genome activation, i.e. at late 8c. The arrest of *CENPF*-mRNA-depleted embryos just at the 8c suggests that until then, the embryos utilize maternal reserves of the protein. In addition, this is in agreement with our CHX experimental data.

In the *CENPF* dsRNA injected 16c embryos, the *CENPF* mRNA was silenced too. This suggests that embryos are to some extent able to compensate the decreased level of *CENPF* mRNA. The depletion of CENPF in somatic cells causes incorrect distribution of chromosomes during mitosis, since the cells form an interaction that is too weak between kinetochores and microtubules (Holt *et al.* 2005, Yang *et al.* 2005, Feng *et al.* 2006). Some of the CENPF-depleted cells progress through mitosis without chromosome segregation in anaphase or form a tripolar spindle resulting in multinucleated cells or aneuploidies (Holt *et al.* 2005,

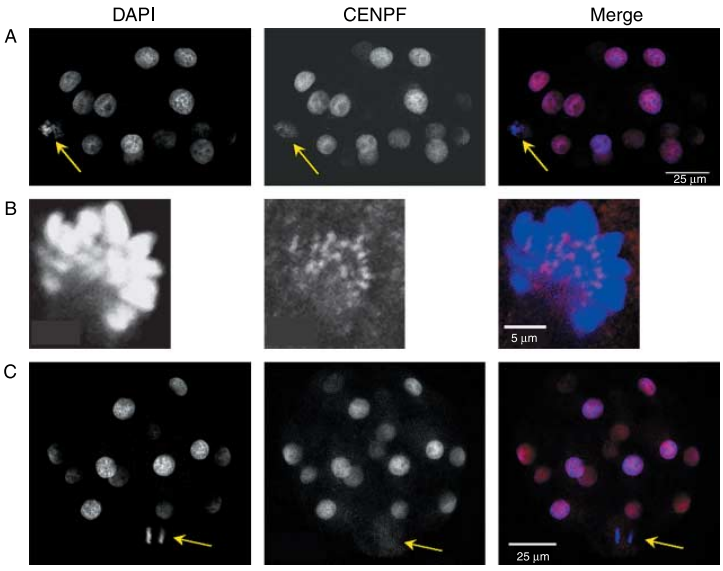


**Figure 7** Localization of CENPF protein in a non-treated bovine preimplantation embryo throughout the cell cycle. (A) and (C) whole embryos, (B) detail of nucleus of a single blastomere in prometaphase; (A) localization on kinetochores in prophase (indicated by arrowhead); (B) localization on kinetochores in prometaphase; (C) degradation of the protein at the end of mitosis (indicated by arrowhead). During interphase CENPF localizes to nucleoplasm (unmarked blastomeres of embryos in (A) and (C). The embryo in (A) and the embryo in (C) were stained using specific mouse anti-CENPF antibody against N-terminus; the single blastomere in (B) was stained using rabbit anti-CENPF antibody against C-terminus. All embryos are early 16-cell stage embryos.

Feng *et al*. 2006). Most of the CENPF-depleted cells do not continue the cell-cycle progress (Holt *et al*. 2005, Yang *et al*. 2005) and undergo apoptosis (Yang *et al*. 2005). However, a minority of cells are able to progress through the cell cycle despite the premature mitotic exit with unaligned chromosomes (Holt *et al*. 2005). The progress of some of the cells is probably caused by insufficient function of the mitotic checkpoint, which is able to delay, but not arrest, the progress (Feng *et al*. 2006). From our immunofluorescence data it follows that the nuclei of *CENPF* dsRNA injected embryos are in many cases fragmented, or the number of nuclei is less than the number of blastomeres (Fig. 3C). This suggests that also in blastomeres the cell cycle progress is arrested. However, some of the blastomeres are able to develop further. Moreover, the results of different studies are not homogeneous as to the cell fate after CENPF silencing (Holt *et al*. 2005, Laoukili *et al*. 2005, Yang *et al*. 2005, Feng *et al*. 2006). Ma *et al*. (2006) suggest that this may be caused by different efficiencies of the mRNA silencing. We, however, agree with the hypothesis of Feng *et al*. (2006), who assumed that the variation in results might be caused by the usage of different cell lines. Since the embryonic cells are strongly forced to cell division, they are supposed to surmount the cell-cycle-arrest signals quite easily.

In conclusion, we showed that the introduction of *CENPF*-specific dsRNA into the zygote leads to mRNA and protein silencing in preimplantation development. The inhibition of *CENPF* mRNA results in considerable deterioration in developmental competence after achieving the 8c and arrest of the majority of embryos before reaching the 16c. These findings are in agreement with data acquired on human somatic cells and indicate that after activation of embryonic genome transcription, CENPF is expressed and localized in the same way as in human somatic cells and that the expression of *CENPF* mRNA is necessary for proper course of preimplantation development.

## Materials and Methods

### *IVF and embryo culture*

Unless otherwise indicated, chemicals were purchased from Sigma (Sigma–Aldrich) and plastic from Nunclon (Nunc, Roskilde, Denmark).

Bovine embryos were obtained after *in vitro* maturation of oocytes and their subsequent fertilization and culture *in vitro*. Briefly, abattoir derived ovaries from cows and heifers were collected and transported in thermo containers in sterile saline at about 33 °C. The follicles with diameter between 5 and 10 mm were dissected with fine scissors and then punctured. The cumulus–oocyte complexes were evaluated and selected according to the morphology of cumulus and submitted to *in vitro* maturation in TCM 199 supplemented with 20 mM sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin, 10% estrus cow serum (ECS) and gonadotropins (P.G. 600, 15 U/ml; Intervet, Boxmeer, Holland) without oil overlay in 4-well dishes under atmosphere of 5% $CO_2$–7% $O_2$–88% $N_2$ at 39 °C for 24 h.

For IVF, the cumulus–oocyte complexes were washed four times in PBS and once in fertilization medium (TALP) and transferred in groups of up to 40 into 4-well dishes containing 250 µl of TALP per well. The TALP medium contained 1.5 mg/ml BSA, 30 µg/ml heparin, 0.25 mM sodium pyruvate, 10 mM lactate and 20 µM penicillamine. Cumulus–oocyte complexes were then co-incubated with frozen–thawed wash

semen from one bull previously tested in the IVF system. Viable spermatozoa were washed in TALP and centrifuged at 100 ***g*** for 5 min. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of TALP to give a concentration of $2\times10^6$ spermatozoa/ml. A 250 µl aliquot of this suspension was added to each fertilization well to obtain a final concentration of $1\times10^6$ spermatozoa/ml. Plates were incubated for 20 h at 39 °C under an atmosphere composed of 5% $CO_2$–7% $O_2$–88% $N_2$.

At 20 h post fertilization (hpf) zygotes were denuded by gentle pipetting, and transferred to B2 Menezo medium supplemented with 10% ECS and cultured in an atmosphere of 5% $CO_2$–7% $O_2$–88% $N_2$ at maximum humidity (25 zygotes in 25 µl of medium under mineral oil; COOK, Eight Mile Plains, Queensland, Australia). The dishes were examined at 32, 44, 56, 92 and 120 hpf and 2-cell, 4-cell, early 8-cell, late 8-cell embryo and morula were collected at each time point respectively.

### *The CHX treatment*

To block the protein synthesis CHX (Sigma–Aldrich) was added to the culture medium at a final concentration of 10 µg/ml 48 and 80 hpf respectively. After 24 h cultivation, the embryos were washed in PBS and fixed for immunofluorescence. In total, 84 embryos were included in the study in four independent experiments – 48 hpf: 27 CHX-treated embryos and 16 controls; 80 hpf: 22 CHX-treated embryos and 19 controls were immunofluorescently examined.

### *Synthesis of DNA template*

The RNA for DNA template synthesis was isolated from bovine embryonic fibroblasts using RNeasy Mini Kit (Qiagen). The template was synthesized using primers '*CENPF* dsRNA' (see Table 1). The identity of fibroblastic and embryonic sequence was verified by sequencing. These primers generated amplicons corresponding to the bovine cDNA sequences in GenBank (XM_612376) and were fused with the T7 promoter. The RT was performed at 55 °C using RETROscript (Ambion, Austin, TX, USA), primed with random decamers. The PCR reaction was performed using SuperTaq polymerase (Ambion). The samples were heated at 95 °C for 3 min followed by 30 cycles of 94 °C 20 s, 50 °C 20 s and 72 °C 45 s. The final extension step was held for 5 min at 72 °C. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen) and the identity was confirmed by sequencing.

### *Synthesis of dsRNA*

The DNA template coupled with T7 promoter was *in vitro* transcribed using MEGAscript RNAi Kit (Ambion). An amount of 1 µg of DNA template was used for each reaction. The reaction mixture was incubated for 5 h at 37 °C and the sense and antisense strands were transcribed in the same reaction. To anneal them, the sample was incubated at 75 °C for 5 min and then left to cool at room temperature. The residual DNA template and ssRNA were digested and the dsRNA was purified according to the manufacturer's instruction. One microlitre of RNA acquired by *in vitro* transcription and 1 µl of final dsRNA were resolved by electrophoresis on 1.5% agarose gel to confirm the integrity of the dsRNA and efficiency of the annealing step.

### *Zygote microinjection*

Good quality zygotes were injected 20 hpf at the stage of two pronuclei. dsRNAs were dissolved in RNase-free water to a final concentration of 800 ng/µl. Zygotes were microinjected with ~5 pl of the dsRNA using an MIS-5000 micromanipulator (Burleigh, Exfo Life Sciences, Mississauga, Ontario, Canada) and PM 2000B4 microinjector (MicroData Instrument, South Plainfield, NJ, USA). Pipettes for microinjection were made using P97 Pipette Puller (Sutter Instrument Company, Novato, CA, USA). Two control groups were established – the uninjected group and a group injected with *GFP* dsRNA. The microinjection medium was Whitten's medium supplemented with 10 mmol/l HEPES (pH 7.3).

In total, 839 embryos were included in the study in 12 independent injection sessions. Embryos were categorized into the following groups: 1) embryos injected with *CENPF* dsRNA (266 embryos), 2) embryos injected with *GFP* dsRNA (237 embryos), and 3) uninjected embryos (336 embryos).

**Table 1** Details of primers used for quantitative RT-PCR and double-stranded RNA synthesis.

| Primer | Sequences | **Annealing temperature** (°C) | **Amplicon size** (bp) |
|---|---|---|---|
| *CENPF* (XM_612376) dsRNA | 5′ AGGATCCTAATACGACTCACTATAGGGA-GAGGGGCTTCCAGAAGTTGTAAA 3′<br>5′ ACTCGAGTAATACGACTCACTATAGGCA-GATGGACCCTACAGTTCTCGCT 3′ | 50 | 413 |
| *GFP* dsRNA[a] (Anger *et al.* 2005) | 5′ AGGATCCTAATACGACTAACTATAGGGA-GAATGGTGAGCAAGGGCGAGGA 3′<br>5′ ACTCGAGTAATACGACTCACTATAGGGA-GAGCGGCCGCTTTACTTGTACA 3′ | 55 | 712 |
| *CENPF* (XM_612376) mRNA quantification | 5′ TTGTAAAGAAAGGGTTTGC 3′<br>5′ CCAGCTGTTGGTTTGGAGG 3′ | 50 | 172 |
| *NPM1* (XM_001252818) mRNA quantification | 5′ ACAGCCAACGGTTTCTCTTG 3′<br>5′ TTTCACCTCCTCCTCCTCCT 3′ | 55 | 154 |
| *H2AFZ* (NM_174809) mRNA quantification | 5′ AGGACGACTAGCCATGGACGTGTG 3′<br>5′ CCACCACCAGCAATTGTAGCCTTG 3′ | 60 | 208 |

[a]Transcribed from empty p-Bluescript-GFP vector; kindly donated by M Anger and P Šolc.

After microinjection, embryos were cultivated under the conditions mentioned above and collected at specific developmental stages (late 8c – day 3.5 post fertilization, late 16c – day 4.5 post fertilization). The number of embryos that reach each developmental stage was counted and the morphological state of each embryo was determined using phase-contrast technique.

### *Monitoring of mRNA degradation efficiency*

The embryos were washed and lysed using FastLane Cell SYBR Green Kit (Qiagen). Single embryos were washed in DMEM without foetal bovine serum and supplemented with 2% (w/v) polyvinylalcohol (PVA), PBS supplemented with 2% (w/v) PVA and FCW buffer (a component of FastLane Cell SYBR Green Kit) and stored dry and deep-frozen at −80 °C until used. Whole single embryos were lysed in 10 µl of the mixture of Buffer FCPL and gDNA Wipeout Buffer 2 (both members of FastLane Cell RT-PCR kit; Qiagen) according to the manufacturer's instructions and the lysate was directly used for the RT-PCR. Quantitative RT-PCR was performed using One Step RT-PCR kit (Qiagen). The samples were incubated at 50 °C for 30 min and heated at 95 °C followed by 45 cycles of 94 °C 20 s, 50 °C 20 s and 72 °C 30 s. The final extension step was held for 10 min at 72 °C. The RT-PCR data were normalized to the number of blastomeres.

The experiments were carried out on Rotor-Gene 3000 (Corbett Research, Mortlake, New South Wales, Australia). Fluorescence data were acquired at 3 °C below the melting temperature to distinguish the possible primer dimers. The qRT-PCR data were determined using serial dilutions; the standard curve was created by Internal Rotor-Gene software (Corbett Research, Mortlake, Australia). The starting amount of corresponding RNA in analysed samples was determined by appointing the Cts to the curve. Products were verified by melting analysis and gel electrophoresis on 1.5% agarose gel with ethidium bromide staining. The experiment was repeated four times.

### *Immunofluorescence*

Embryos were fixed in 4% paraformaldehyde supplemented with 1% (v/v) Triton X-100 for 50 min at 4 °C. Fixed embryos were processed immediately or stored in PBS up to 3 weeks at 4 °C. After washing in PBS embryos were incubated in 1% (v/v) Triton X-100 for 15 min. All subsequent steps were done in PBS supplemented with BSA (0.25% for mouse anti-CENPF antibody against N-terminus – BD Biosciences, Erembodegem, Belgium; 0.4% for rabbit anti-CENPF antibody against C-terminus – Novus Biologicals, Littleton, CO, USA) and 0.05% (w/v) saponin (PBS/BSA/sap). Embryos were blocked with 2% (v/v) normal goat serum for 1 h and incubated with primary antibody in PBS/BSA/sap overnight at 4 °C (mouse anti-CENPF antibody against N-terminus – 1:100; rabbit anti-CENPF antibody against C-terminus – 1:1000). After thorough washing the embryos were incubated with goat anti-mouse antibody conjugated with Alexa 594 (Invitrogen) or goat anti-rabbit antibody conjugated with FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with Alexa 594 (Invitrogen) in PBS/BSA/sap for 1 h at room temperature in the dark. The nuclei were stained and the embryos were mounted on glass slides using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). The samples were examined with a confocal laser-scanning microscope Leica TCS SP (Leica Microsystems AG, Wetzlar, Germany). Controls of immunostaining specifity were carried out by omitting primary antibody or using another species-specific secondary antibody conjugate. The images were processed using the ImageJ software (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij).

### *Statistical analyses*

The data were analysed using SigmaStat 3.0 software (Jandel Scientific, San Rafael, CA, USA), the Student's *t*-test or Mann–Whitney Rank Sum tests were used. $P<0.05$ was considered as significant.

## Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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## References

**Adjaye J, Herwig R, Brink TC, Herrmann D, Greber B, Sudheer S, Groth D, Carnwath JW, Lehrach H & Niemann H** 2007 Conserved molecular portraits of bovine and human blastocysts as a consequence of the transition from maternal to embryonic control of gene expression. *Physiological Genomics* **31** 315–327.

**Anger M, Stein P & Schultz RM** 2005 CDC6 requirement for spindle formation during maturation of mouse oocytes. *Biology of Reproduction* **72** 188–194.

**Ashe M, Pabon-Pena L, Dees E, Price KL & Bader D** 2003 LEK is a potential inhibitor of pocket protein-mediated cellular processes. *Journal of Biological Chemistry* **279** 664–676.

**Bilodeau-Goeseels S & Schultz GA** 1997 Changes in ribosomal ribonucleic acid content within *in vitro*-produced bovine embryos. *Biology of Reproduction* **56** 1323–1329.

**Bolton VN, Oades PJ & Johnson MH** 1984 The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. *Journal of Embryology and Experimental Morphology* **79** 139–163.

**Bomont P, Maddox P, Shah JV, Desai AB & Cleveland DW** 2005 Unstable microtubule capture at kinetochores depleted of the centromere-associated protein CENPF. *EMBO Journal* **24** 3927–3939.

**Camous S, Kopecny V & Flechon JE** 1986 Autoradiographic detection of the earliest stage of [$^3$H]-uridine incorporation into the cow embryo. *Biology of the Cell* **58** 195–200.

**Cheeseman IM, MacLeod I, Yates JR III, Oegema K & Desai A** 2005 The CENP-F-like proteins HCP-1 and HCP-2 target CLASP to kinetochores to mediate chromosome segregation. *Current Biology* **5** 771–777.

**DeBaun MR, Niemitz EL & Feinberg AP** 2003 Association of *in vitro* fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *American Journal of Human Genetics* **72** 156–160.

**Dees E, Robertson JB, Ashe M, Pabon-Pena L, Bader D & Goodwin RL** 2005 LEK1 protein expression in normal and dysregulated cardiomyocyte mitosis. *Anatomical Record. Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology* **286** 823–832.

**Ecker DJ, Stein P, Xu Z, Williams CJ, Kopf GS, Bilker WB, Abel T & Schultz RM** 2004 Long-term effects of culture of preimplantation mouse embryos on behavior. *PNAS* **101** 1595–1600.

**Evans HJ, Edwards L & Goodwin RL** 2007 Conserved C-terminal domains of mCenpf (LEK1) regulates subcellular localization and mitotic checkpoint delay. *Experimental Cell Research* **313** 2427–2437.

**Feng J, Huang H & Yen TJ** 2006 CENPF is a novel microtubule-binding protein that is essential for kinetochores attachments and affects the duration of the mitotic delay. *Chromosoma* **115** 320–329.

**Goodwin RL, Pabón-Peña LM, Foster GC & Bader D** 1999 The cloning and analysis of LEK1 identities variations in the LEK/centromere protein F/mitosin gene family. *Journal of Biological Chemistry* **274** 18597–18604.

**Hajeri VA, Stewart AM, Moore LL & Padilla PA** 2008 Genetic analysis of the spindle checkpoint genes san-1, mdf-2, bub-3 and the CENP-F homologues hcp-1 and hcp-2 in *Caenorhabditis elegans*. *Cell Division* **3** 6.

**Hales CN & Barker DJP** 2001 The thrifty phenotype hypothesis. *British Medical Bulletin* **60** 5–20.

**Hamatani T, Carter MG, Sharov AA & Ko MSH** 2004 Dynamics of global gene expression changes during mouse preimplantation development. *Developmental Cell* **6** 117–131.

**Holt SV, Vergnolle MA, Hussein D, Wozniak MJ, Allan VJ & Taylor SS** 2005 Silencing Cenpf weakens centromeric cohesion, prevents chromosome alignment and activates the spindle checkpoint. *Journal of Cell Science* **18** 4889–4900.

**Hussein D & Taylor SS** 2002 Farnesylation of Cenpf is required for G2/M progression and degradation after mitosis. *Journal of Cell Science* **115** 3403–3414.

**Iwamori N, Naito K, Sugiura K & Tojo H** 2002 Preimplantation-embryo-specific cell cycle regulation is attributed to the low expression level of retinoblastoma protein. *FEBS Letters* **526** 119–123.

**Jakobsen AS, Avery B, Dieleman SJ, Knijn HM, Vos PLAM & Thomsen PD** 2006 Transcription of ribosomal RNA genes is initiated in the third cell cycle of bovine embryos. *Molecular Reproduction and Development* **73** 196–205.

**Kanka J, Kepkova K & Nemcova L** 2009 Gene expression during minor genome activation in pre-implantation bovine development. *Theriogenology* **72** 572–583.

**King WA, Niar A, Chartrain I, Betteridge KJ & Guay P** 1988 Nucleolus organizer regions and nucleoli in preattachment bovine embryos. *Journal of Reproduction and Fertility* **82** 87–95.

**Kopecny V, Flechon JE, Camous S & Fulka J Jr** 1989 Nucleologenesis and the onset of transcription in the eight-cell bovine embryo: fine-structural autoradiographic study. *Molecular Reproduction and Development* **1** 79–90.

**Kues WA, Sudheer S, Herrmann D, Carnwath JW, Havlicek V, Besenfelder U, Lehrach H, Adjaye J & Niemann H** 2008 Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development *in vivo*. *PNAS* **105** 19768–19773.

**Laoukili J, Kooistra MR, Bras A, Kauw J, Kerkhoven RM, Morrison A, Clevers H & Medema RH** 2005 FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nature Cell Biology* **7** 126–136.

**Liao H, Winkfein RJ, Mack G, Rattner JB & Yen TJ** 1995 CENPF is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. *Journal of Cell Biology* **130** 507–518.

**Ma L, Zhao X & Zhu X** 2006 Mitosin/CENP-F in mitosis, transcriptional control, and differentiation. *Journal of Biomedical Science* **13** 205–213.

**Memili E & First NL** 2000 Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8** 87–96.

**Misirlioglu M, Page GP, Sagirkaya H, Kaya A, Parrish JJ, First NL & Memili E** 2006 Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. *PNAS* **103** 18905–18910.

**Nganvongpanit K, Müller H, Rings F, Gilles M, Jennen D, Hölker M, Tholen E, Schellander K & Tesfaye D** 2006*a* Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. *Molecular Reproduction and Development* **73** 153–163.

**Nganvongpanit K, Müller H, Rings F, Hölker M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Schellander K & Tesfaye D** 2006*b* Selective degradation of maternal and embryonic transcripts in *in vitro* produced bovine oocytes and embryos using sequence specific double-stranded RNA. *Reproduction* **131** 861–874.

**Pavlok A, Kopecny V, Lucas-Hahn A & Niemann H** 1993 Transcriptional activity and nuclear ultrastructure of 8-cell bovine embryos developed by *in vitro* maturation and fertilization of oocytes from different growth categories of antral follicles. *Molecular Reproduction and Development* **35** 233–243.

**Pouwels J, Kukkonen M, Lan W, Daum JR, Gorbsky GJ, Stukenberg T & Kallio MJ** 2007 Shugoshin 1 plays a central role in kinetochore assembly and is required for kinetochore targeting of Plk1. *Cell Cycle* **6** 1579–1585.

**Redkar A, deRiel JK, Xu Y-S, Montgomery M, Patwardhan V & Litvin J** 2002 Characterization of cardiac muscle factor 1 sequence motifs: retinoblastoma protein binding and nuclear localization. *Gene* **282** 53–64.

**Soukolis V, Reddy S, Pooley RD, Feng Y, Walsh CA & Bader DM** 2005 Cytoplasmic LEK1 is a regulator of microtubule function through its interaction with LIS1 pathway. *PNAS* **102** 8549–8554.

**Telford NA, Watson AJ & Schultz GA** 1990 Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Molecular Reproduction and Development* **26** 90–100.

**Vigneault C, Gravel C, Vallee M, McGraw S & Sirard MA** 2009 Unveiling the bovine embryo transcriptome during the maternal to embryonic transition. *Reproduction* **137** 254–257.

**Wang QT, Piotrowska K, Ciemerych MA, Milenkovic L & Zernicka-Goetz M** 2004 A genome-wide study of gene activity reveals developmental signalling pathways in the preimplantation mouse embryo. *Developmental Cell* **6** 133–144.

**Wei Y, Bader D & Litvin J** 1996 Identification of a novel cardiac-specific transcript critical for cardiac myocyte differentiation. *Development* **122** 2779–2789.

**Yang Z, Guo J, Chen Q, Ding C, Du J & Zhu X** 2005 Silencing mitosin induces misaligned chromosomes, premature chromosome decondensation before anaphase onset, and mitotic cell death. *Molecular and Cellular Biology* **25** 4062–4074.

**Zhou X, Wang R, Fan L, Li Y, Ma L, Yang Z, Yu W, Jing N & Zhu X** 2005 Mitosin/CENPF as a negative regulator of activating transcription factor-4. *Journal of Biochemistry* **280** 13973–13977.

**Zhu X, Mancini MA, Chang K-H, Liu C-Y, Chen C-F, Shan B, Jones D, Yang-Feng TL & Lee W-H** 1995 Characterization of a novel 350-kilodalton nuclear phosphorotein that is specifically involved in mitotic-phase progression. *Molecular and Cellular Biology* **15** 5017–5029.

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Abstract: Pre-implantation embryos derived by in vitro fertilization differ in their developmental potential from embryos obtained in vivo. In order to characterize changes in gene expression profiles caused by in vitro culture environment, we employed microarray constructed from bovine oocyte and preimplantation embryo-specific cDNAs (BlueChip, Université Laval, Québec). The analysis revealed changes in the level of 134 transcripts between in vitro derived (cultured in COOK BVC/BVB media) and in vivo derived 4-cell stage embryos and 97 transcripts were differentially expressed between 8-cell stage in vitro and in vivo embryos. The expression profiles of 7 selected transcripts (BUB3, CUL1, FBL, NOLC1, PCAF, GABPA and CNOT4) were studied in detail. We have identified a switch from Cullin 1-like transcript variant 1 to Cullin 1 transcript variant 3 (UniGene IDs BT.36789 and BT.6490, respectively) expressions around the time of bovine major gene activation (8-cell stage). New fibrillarin protein was detected by immunofluorescence already in early 8-cell stage and this detection correlated with increased level of fibrillarin mRNA. The qRT-PCR analysis revealed significant differences in the level of BUB3, NOLC1, PCAF, GABPA and CNOT4 gene transcripts between in vivo derived (IVD) and in vitro produced (IVP) embryos in late 8-cell stage. The combination of these genes represents a suitable tool for addressing questions concerning normal IVD embryo development and can be potentially useful as a marker of embryo quality in future attempts to optimize in vitro culture conditions.

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Dear Mrs. Vodickova Kepkova,

I am pleased to confirm that your manuscript, "Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition., has been accepted for publication in Theriogenology.

The files have been forwarded to the publisher and you will be contacted in due course regarding galley proofs.

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Sincerely,

Fulvio Gandolfi, DVM, PhD
Co-Editor-in-Chief
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# Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition.

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## Abstract

Pre-implantation embryos derived by *in vitro* fertilization differ in their developmental potential from embryos obtained *in vivo*. In order to characterize changes in gene expression profiles caused by *in vitro* culture environment, we employed microarray constructed from bovine oocyte and preimplantation embryo-specific cDNAs (BlueChip, Université Laval, Québec). The analysis revealed changes in the level of 134 transcripts between *in vitro* derived (cultured in COOK BVC/BVB media) and *in vivo* derived 4-cell stage embryos and 97 transcripts were differentially expressed between 8-cell stage *in vitro* and *in vivo* embryos. The expression profiles of 7 selected transcripts (*BUB3, CUL1, FBL, NOLC1, PCAF, GABPA* and *CNOT4*) were studied in detail. We have identified a switch from Cullin 1-like transcript variant 1 to Cullin 1 transcript variant 3 (UniGene IDs **BT.36789** and **BT.6490**, respectively) expressions around the time of bovine major gene activation (8-cell stage). New fibrillarin protein was detected by immunofluorescence already in early 8-cell stage and this detection correlated with increased level of fibrillarin mRNA. The qRT-PCR analysis revealed significant differences in the level of *BUB3*, *NOLC1*, *PCAF*, *GABPA* and *CNOT4* gene transcripts between *in vivo* derived (IVD) and *in vitro* produced (IVP) embryos in late 8-cell stage. The combination of these genes represents a suitable tool for addressing questions concerning normal IVD embryo development and can be potentially useful as a marker of embryo quality in future attempts to optimize *in vitro* culture conditions.

*Keywords:* preimplantation embryo; *in vivo* derived; microarray; real-time RT-PCR; Cullin 1; fibrillarin

## Abbreviations

| | |
|---|---|
| aRNA | Amplified RNA |
| BVB | Bovine Vitro Blast medium |
| BVC | Bovine Vitro Cleave medium |
| DAPI | 4',6-diamidino-2-phenylindole |
| DFC | Fibrillar component |
| EST | Expressed sequence tag |
| FC | Fibrillar centre |
| FDR | False discovery rate |
| GV | Germinal vesicle stage oocyte |
| hpf | Hours post fertilization |
| I2D | Interologous Interaction Database |
| IETS | International Embryo Transfer Society |
| IVD | In vivo derived |
| IVP | In vitro produced |
| lowess | Locally weighted scatterplot smoothing |
| MET | Maternal-to-embryonic transition |
| MII | Metaphase II stage oocyte |
| MPM | Modified Parker medium |
| NAViGaTOR | Network Analysis, Visualization & Graphing TORonto |
| qRT-PCR | Quantitative RT-PCR |
| SAM | Significance analysis of microarrays |
| sap | Saponin |
| SDS | Sodium dodecyl sulfate |
| SSC | Saline sodium citrate buffer |
| SSH | Suppression Subtractive Hybridization |

## 1. Introduction

*In vitro* produced (IVP) bovine embryos represent a valuable resource for embryology research and recently also for routine embryo transfer, but IVP process still suffers from low efficiency (<40% IVP embryos reaching blastocyst stage). Factors contributing to this problem include oocyte quality, conditions of *in vitro* oocyte maturation and embryo culture conditions [1]. To address these problems, several groups have studied the influence of different media compositions and culture conditions on the developmental competence of *in vitro* cultured bovine oocytes [2,3,4]. Others have concentrated on embryonic preimplantation development period where maternal-to-embryonic transition (MET) takes place. This shift from utilization of maternally produced and stored transcripts to mRNAs produced by newly activated embryonic genome occurs at a species-specific time-point [5]. In the bovine embryos, a minor gene activation was reported to start between 1-4-cell stage [6], while major gene activation takes place at 8-16-cell stage [7]. Attempts were made to compare changes in abundance of specific transcripts during this developmental period and to relate them to the quality of IVP and *in vivo* derived (IVD) bovine embryos. Gutierrez-Adan et al. [8] have shown that stress response genes are up-regulated during *in vitro* development while metabolism related genes are down-regulated compared to *in vivo* derived embryos. For some markers (SOX, G6PD) changes were detected before MET, suggesting possible influence of *in vitro* culture on the rate of maternal mRNA stocks polyadenylation or depletion/degradation. The disadvantage of similar quantitative RT-PCR (qRT-PCR) based studies is the fact that the levels of only a few preselected transcripts could be studied. Alternative strategies allowing for wider transcriptome coverage include screening of stage specific EST libraries [9] or use of microarrays [10,11]. The previously cited studies compared IVD embryos or oocytes with IVP embryos at the blastocyst stage which occurs several days after MET at the 8-cell stage [10,11]. A study by Vigneault et al. [12] showed that many transcripts are newly expressed already in the 6-cell and early 8-cell stage bovine embryos. Thus we have utilized custom bovine embryo-specific microarray (BlueChip, [13]) to characterize transcriptome changes between IVP and IVD bovine embryos at the time of minor and major embryonic genome activation (4-cell stage and 8-cell stage, respectively). Based on a list of candidate genes identified by microarray, we have studied the expression levels of selected transcripts during *in vitro* culture of preimplantation embryos in different culture media.

## 2. Materials and methods

### 2.1. Isolation of bovine oocytes, *in vitro* fertilization and embryo culture

Bovine embryos were obtained after *in vitro* maturation of oocytes and their subsequent fertilization and culture *in vitro* [14]. Antral follicles with diameter between 4 and 10 mm were dissected with fine scissors and then punctured. The cumulus-oocyte complexes were evaluated and selected according to the morphology of cumulus and submitted to in vitro maturation in tissue culture medium (TCM) 199 supplemented with 20 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% estrus cow serum (ECS) and gonadotropins

(P.G. 600, 15 U/ml, Intervet, Boxweer, Netherlands) without oil overlay in 4 –well dishes under atmosphere of 5% $CO_2$ – 7% $O_2$ – 88% $N_2$ at 39˚C for 24 h.

For *in vitro* fertilization (IVF), cumulus-oocyte complexes were washed four times in PBS and once in fertilization medium, then transferred in groups of up to 40 into four-well dishes (Nunc) containing 250 µl of fertilization medium [14] per well. Viable spermatozoa were washed in fertilization medium and pelleted by centrifugation at 100 x g for 5 min. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of fertilization medium to give a concentration of 2 x $10^6$ spermatozoa/ml. A 250 µl aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1 x $10^6$ spermatozoa/ml. Plates were incubated for approximately 20 h at 39 ˚C in an atmosphere composed of 5% $CO_2$ - 7% $O_2$ - 88% $N_2$.

At approximately 20 h post fertilization (hpf) presumptive zygotes were denuded by gentle pipetting and divided into two groups. The first group were transferred to Bovine Vitro Cleave medium (BVC, COOK, Eight Mile Plains, Australia) and the second group were transferred to Menezo B2 medium (Sevapharma, Prague, Czech Republic). All zygotes were cultured in an atmosphere of 5% $CO_2$ - 7% $O_2$ - 88% $N_2$ at maximum humidity (25 zygotes in 25 µl of medium under mineral oil). At 100 hpf the BVC medium was replaced by Bovine Vitro Blast medium (BVB, COOK) and both groups of embryos were cultured to the hatched blastocyst stage. The dishes were examined at 32, 44, 60, 92, 120, 156 and 180 hpf, and 2-cell, 4-cell, early 8-cell, late 8-cell embryo, morula, blastocysts and hatched blastocysts were collected at each respective time point.

**2.2. Recovery of embryos at different developmental stages from bovine oviducts**

Hormonal treatment of donor animals and embryo collection were performed according to [15]. Briefly, a total of 24 heifers (crossbreed Czech Motley and Holstein) were pre-synchronized by administration of prostaglandin F2α analogue (cloprostenol 500 µg i.m., Oestrophan® 0.25 mg/ml Bioveta, Czech Republic) twice within 11 days. Two days after each of the PGF2α treatments the animals received GnRH analogue (lecirelin 50 µg i.m., Supergestran®,Nordic Pharma, Czech Republic). Ultrasound-guided transvaginal aspirations of the dominant follicles were performed on day 9 of estrus cycle. Forty two hours later the animals received the first of eight consecutive FSH-injections (in total 450 UI of FSH at 12 h intervals in decreasing doses, Pluset®, Calier S.A., Spain). Two PGF2α treatments were performed 60 and 72 h after the initial FSH-treatment. Finally, 48 hours after the first PGF2α treatment, ovulation was induced by giving hCG (2500 UI i.v., Pregnyl®1500, Organon, Netherlands) simultaneously with the artificial insemination (AI) using one straw of frozen semen from a bull of proven fertility. Embryos at 2-cell, 4-cell and late 8-cell stage were recovered by oviductal flushing using a minimally invasive endoscopic approach 1-4 days after artificial insemination [16]. The developmental stage as well as the morphological integrity of the recovered embryos was assessed according to IETS guidelines [17]. Embryos of the same developmental stage assessed excellent and good were pooled in groups of ten embryos, washed in PBS, frozen in a minimum amount of medium in siliconized 0.6 ml cups and

stored at −80 °C. All animal treatments were approved by the Central Committee for Animal Protection, Ministry of Agriculture of the Czech Republic, Prague (project of experiments No. 37/2006) and fully conformed to the Czech Animal Protection Law (No. 246/92).

**2.3. Suppression subtractive hybridization and cDNA array preparation**

PolyA+ mRNA from pools of 25 *in vitro* matured metaphase II oocytes or 50 4-cell stage and 8-cell stage *in vitro* produced embryos after culture in COOK BVC/BVB medium was extracted by a Dynabeads mRNA DIRECT Micro Kit (Dynal, Oslo, Norway) according to the manufacturer's instructions.

The extracted mRNA was converted into cDNA and amplified using Super Smart cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, California, USA) and subtraction immediately followed using PCR Select cDNA Subtraction Kit (BD Biosciences Clontech, Palo Alto, California, USA) according to the manufacturer's instructions. *In vitro* produced 4-cell stage embryos were subtracted from IVP 8-cell stage embryos, IVP 8-cell stage embryos from IVP 4-cell stage embryos and *in vitro* matured MII stage oocytes from IVP 4-cell stage embryos. Selected subtracted cDNAs were sent to the Centre de Recherche en Biologie de la Reproduction, Département de Sciences Animales, Université Laval, where they were used for microarray preparation.

BlueChip, V3 array contains 3136 sequences spotted in triplicates (9 408 spots in total, including controls). All experimental sequences were derived from cDNA libraries generated by the set of SSHs with bovine oocytes, preimplantation embryos and somatic tissues: GV oocytes subtracting somatic tissues, GV oocytes subtracting day 8 blastocysts, day 8 blastocyst subtracting GV oocytes, day 8 blastocyst subtracting somatic tissues (SSH libraries present on the original BlueChip array as described in [13]), 4-cell stage embryos subtracting MII oocytes, 4-cell stage embryos subtracting 8-cell stage embryos, 8-cell stage embryos subtracting 4-cell stage embryos (3 libraries generated in our laboratory, as described above), 8-cell stage embryos subtracting **α**-amanitin treated 8-cell stage embryos, early cleaving 2-cell stage embryos subtracting late cleaving embryos, 6 h *in vitro* matured oocytes subtracting GV oocytes, 6 h *in vivo* matured oocytes subtracting 6 h *in vitro* matured oocytes and 6 h post-LH *in vivo* oocytes subtracting 2 h pre-LH *in vivo* oocytes. Samples coming from the SpotReport Alien cDNA Array Validation System (Stratagene, La Jolla, USA), Alien 1 (540 spots) and Alien 2 (540 spots) and other samples, namely blank (96 spots), GFP (4 spots), GFP1 (378 spots), GFP1 (6 spots), GFP1/16 (6 spots), GFP1/8 (6 spots), GFP L (6 spots) and H20/DMSO (384 spots), negative (6 spots), plant (540 spots) were included in the array as negative controls. These spots can be used for the determination of background hybridization during statistical analysis. Housekeeping genes, namely tubulin (12 spots), ubiquitin (12 spots), and actin (12 spots) were added as positive controls.

**2.4. RNA extraction and aRNA preparation**

One biological replicate represented by a pool of 20 embryos was prepared for each stage studied (8-cell stage IVD and IVP, 4-cell stage IVD and IVP embryos) and three technical replicates including dye-swap were performed for each comparison.

Poly (A)+ mRNA was extracted from pools of 20 *in vivo* or *in vitro* (after culture in COOK BVC/BVB medium) produced 4-cell and 8-cell stage embryos, using a Dynabeads mRNA DIRECT Micro Kit (Dynal, Oslo, Norway) according to the manufacturer's instructions.

Amplified RNA (aRNA) was prepared with a two-round amplification protocol using Amino Allyl MessageAmp ™II aRNA Amplification kit (Ambion, Austin, Texas, USA). In subsequent labelling reaction, 5 μg of aRNA were conjugated with either Alexa Fluor 555 or Alexa Fluor 647 dyes (Invitrogen, Carlsbad, California, USA).

**2.5. Array hybridization, scanning and data analysis**

Labelled probes (2 μg of each Alexa Fluor 555 and 647 conjugated aRNA) were mixed together and denatured for 5 min at 90 °C. Denatured probes were mixed with 45 μl of SlideHyb™ Glass Array Hybridization Buffer #1 (Ambion, Austin, Texas, USA) preheated to 68°C and incubated on the array for 18 hours at 50 °C in a humidified chamber. Slides were washed twice with 2x SSC-0.5% SDS for 15 min at 55 °C, twice with 0.5x SSC-0.5 % SDS for 15 min at 55 °C and dried by centrifugation at room temperature for 5 min at 1200g. Images were scanned using GeneTAC UC4 microarray scanner (Genomic Solutions, Ann Arbor, Michigan, USA) and analyzed using TIGR Spotfinder software 3.1.1 (TM4 software suit, [18]). After within-slide lowess (locally weighted scatterplot smoothing) and between-slide scale normalization [19] in TIGR Midas 2.19, the data were sorted in Excel (Microsoft, Redmond, Washington, USA), and only the features with signal present in all three within-array replicates were selected, averaged and used for further analysis of differential expression in significance analysis of microarrays software ([20], SAM version 3.02, Stanford University; http://www-stat.stanford.edu/~tibs/SAM/). Using one class response type, parameter delta was adjusted so that median number of false positives among differentially expressed genes was <1. This resulted in 134 genes identified as differentially expressed between 4-cell stage IVD and IVP embryos, with FDR of 0.56 % and 97 genes identified as differentially expressed between 8-cell stage IVD and IVP embryos, with FDR of 0.63%. Interologous Interaction Database (I2D; an on-line database of known and predicted mammalian and eukaryotic protein-protein interactions, [21]) was used to search for possible protein-protein interaction partners for proteins identified as regulated in our microarray study. Version 1.80 of I2D, including 254,361 source interactions and 238,288 predicted interactions (http://ophid.utoronto.ca/ophidv2.201/ppi.jsp) was used for interaction search with human selected as target organism. Protein-protein interaction network was visualized using NAViGaTOR (Network Analysis, Visualization & Graphing TORonto) 2.1.13 software (http://ophid.utoronto.ca/navigator). In protein-protein interaction networks, nodes represent proteins and edges between nodes represent physical interactions between the proteins. NAViGaTOR allows nodes to be color-coded according to Gene Ontology (GO - a controlled vocabulary describing properties of genes) terms. "View groups of approximate cliques" analysis tool was used on the resulting network to identify sets of nodes that are highly interconnected (cliques).

**2.6. Quantitative RT-PCR analysis of gene expression**

Poly (A)+ mRNA was extracted from the pools of 20 oocytes and embryos in each stage of development, using a Dynabeads mRNA DIRECT Micro Kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. Before isolation 1 pg of the Luciferase mRNA (Promega, Madison, Wisconsin, USA) per oocyte/embryo was added as an external standard.

Levels of specific mRNAs (selected genes and primer sequences designed using Beacon Designer7 are listed in Table 1) were measured by real-time RT-PCR. mRNA equivalent of 0.5 embryo was amplified by a One-step RT-PCR kit (Qiagen, Hilden, Germany) with real-time detection using SybrGreenI fluorescent dye on a RotorGene 3000 instrument (Corbett Research, Mortlake, Australia). The reaction mix contained QIAGEN OneStep RT-PCR Buffer (1x), dNTP Mix (400 μM of each), forward and reverse primers (both 400 μM), SybrGreenI (1:50.000 of 1000x stock solution, Invitrogen, Carlsbad, California, USA), RNasine inhibitor (Promega, Madison, Wisconsin, USA), RT-PCR Enzyme Mix (1μl) and template RNA. The real-time RT-PCR reactions were prepared in duplicates, with oocytes and embryos on the one reaction, and reactions were repeated three times.

Reaction conditions were: reverse transcription at 50 °C for 30 min, initial activation at 95 °C for 15 min, cycling: denaturation at 94 °C for 20 sec, annealing at a temperature specific for each set of primers (see Table 1) for 20 sec, extension at 72 °C for 30 sec. Products were verified by melting analysis and gel electrophoresis on 1.5 % agarose gel with ethidium bromide staining.

The relative concentration of templates in different samples was determined using comparative analysis software (Corbett Research, Mortlake, Australia). The results for individual target genes were normalized according to the relative concentration of the external standard. Ratios of the target gene concentration to the Luciferase mRNA concentration were estimated in each sample.

Data are presented in as mean ± SEM. The mean ± SEM was obtained from three independent real-time RT-PCRs from three different batches of embryos. The significance of differences between stages 8-cell *in vitro* and 8-cell *in vivo* was evaluated using a t-test (Kyplot v2.0 beta15, KyensLab Incorporated, Tokyo, Japan)

**2.7. Sequencing**

A fragment of the Cullin 1 gene, amplified by PCR with the use of primers listed in Table 1, was purified using a GenElute PCR Clean-Up Kit (Sigma-Aldrich, Prague, Czech Republic). Sequencing reaction was performed with one of the amplification primers (3.2 pmol, forward primer) and 10 ng/100 bp of purified PCR fragment, using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Prague, Czech Republic). Sequence readout was performed on the 3100-Avant Genetic Analyzer (Applied Biosystems, Prague, Czech Republic). Analysis and alignment of resulting sequences was performed in BioEdit 7.0.9.1 and ClustalX 2.0.12 software.

### 2.8. Analysis of CUL1 gene structure

Exalign [22] web interface (http://159.149.109.9/exalign/) was used to compare exonic structure of bovine cullin 1 gene (UniGene **BT.6490**) with the whole set of human, mouse, rat and cow gene structures in a BLAST-like way ("database search" mode), looking for the genes with the most similar structure to the one of the query gene. Bovine cullin 1 mRNA RefSeq ID **XM_876699** and cullin 1-like RefSeq ID **XM_589507** were used as input with following advanced options: Include XM genes: yes; Display top 3 results; Only results with Blast hit: yes.

### 2.9. Immunostaining

Embryos were fixed in 4% paraformaldehyde supplemented with 1% TritonX-100 50 min. at 4°C. Fixed embryos were processed immediately or stored in PBS up to 3 weeks at 4°C. After washing in PBS, the embryos were incubated in 0.75% TritonX-100 for 15 min. All subsequent steps were done in PBS supplemented with 0.25% BSA and 0.05% saponin (PBS/BSA/sap). Embryos were blocked with 2% normal goat serum for 1 hour and incubated with primary antibody (rabbit polyclonal anti-Cullin 1 – Abcam, Cambridge, UK or mouse monoclonal anti-Fibrillarin – Cytoskeleton, Denver, Colorado, USA) 1:100 in PBS/BSA/sap overnight at 4°C. After thorough washing the embryos were incubated with goat anti-mouse antibody conjugated with Alexa 594 (Invitrogen, Carlsbad, California, USA) 1:800 in PBS/BSA/sap or with anti-rabbit fluorescein conjugated antibody 1:350 in PBS/BSA/sap (Santa Cruz biotechnology, Heidelberg, Germany) for 1 hour at room temperature darkling. Controls of immunostaining specificity were carried out by omitting primary antibody or using another species specific secondary antibody conjugate. The nuclei were stained and the embryos were mounted on glass slides using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). Fluorescence was detected on Leica TCS SP2 laser-scanning confocal microscope (Leica, Mannheim, Germany).

## 3. Results and discussion

### 3.1 Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) is a powerful technology for identification of genes that are differentially regulated between two samples. SSH was used to analyze transcription activation during preimplantation development of rabbit and bovine embryos [23]. We have used SSH to prepare three bovine cDNA libraries enriched for transcripts differentially regulated during preimplantation development. Selected cDNAs (Supplementary File 1, labelled in green) representing novel clones, not present on previous version of the bovine oocyte and preimplantation embryo specific BlueChip micrroarray [13] were included in BlueChip version 3 array design.

**3.2 Microarray and protein-protein interaction network modelling**

Due to the small amount of RNA in one embryo and the limited source of IVD embryos, pooling was used to average biological variation and to obtain sufficient starting amount of RNA for amplification. As only one biological replicate represented by a pool of 20 embryos was available for each stage studied, our selection of differentially expressed genes is based on statistical analysis of technical replicates. By this methodology, it is possible to identify genes differentially expressed in the sample particularly studied (e.g. differences between the one particular group of 4-cell stage IVD embryos and the other particular group of 4-cell stage IVP embryos) but not to generalize obtained results to other samples. However, in this case the true independent biological unit should be one embryo or group of embryos from one particular donor and thus the pool of 20 embryos, originated from several donors should represent sufficient sample to warrant biologically meaningful results and to generate the useful list of candidate genes for further study. Significance analysis of microarrays (SAM, [20]) software was used to identify differentially expressed genes. We have identified 81 candidate genes as more abundant and 53 candidate genes as less abundant in 4-cell stage IVP compared to IVD embryos. Another 47 candidate genes more abundant and 50 candidate genes less abundant in 8-cell stage IVP compared to IVD embryos were identified. Complete microarray data including raw data are deposited in Gene Expression Omnibus (GEO), Accession No. **GSE24714**; selected candidate genes are listed in Supplementary File 2.

Where possible, Protein/Swiss-Prot accession numbers of human orthologs to candidate genes identified as differentially regulated by microarray approach were found (Supplementary File 2) and introduced into Interologous Interaction Database (I2D) [21] in order to explore possible interaction partners and to construct protein-protein interaction network (Supplementary Files 3) that enables the graphical visualization of possible functional relationships among molecules (Supplementary File 4, simplified network view in Fig. 1). Four groups of highly interconnected nodes (cliques) were identified in the resulting network by an automated computational algorithm. The first identified cluster contained 5 proteins corresponding to the genes found to be differentially regulated in IVP and IVD embryos: Fibrillarin (FBL), Eukaryotic translation initiation factor 4E (EIF4E), 60S ribosomal protein L5 (RPL5), 60S ribosomal protein L8 (RPL8) and 60S acidic ribosomal protein P0 (RPLP0) (Fig. 1, dark blue lines). The second cluster consisted of three proteins, all corresponding to the genes identified as differentially regulated in IVD vs IVP embryos: Cullin 1 (CUL1), S-phase kinase-associated protein 1A (SKP1A) and Catenin beta-1 (CTNNB1) (Fig. 1, dark red lines). The third cluster contained 2 proteins corresponding to the genes identified as differentially regulated in IVD vs IVP embryos: CCR4-NOT transcription complex subunit 4 (CNOT4) and UHRF2 E3 ubiquitin-protein ligase (UHRF2) and four other interacting proteins (Fig. 1, green lines). From these 3 clusters, we have selected *FBL, CUL1* and *CNOT4* genes for further study. The last cluster contained 2 proteins corresponding to the genes identified as differentially regulated in IVD vs IVP embryos: SUV39H2 Histone-lysine N-methyltransferase (SUV39H2) and SMG1 Serine/threonine-protein kinase (SMG1) and two other interacting

proteins (Fig. 1, light blue lines); no gene from this cluster was selected for further study. Instead, we have selected 4 other transcripts from the list of candidate genes (BUB3, NOLC1, PCAF and GABPA), based on their reported or presumptive role in embryonic development and studied their expression by qRT-PCR.

### 3.3 Expression of selected transcripts in IVP and IVD embryos

For selected transcripts, we have first evaluated if differential expression between IVP and IVD embryos observed on microarray will be confirmed by qRT-PCR. Then we analyzed the expression pattern of selected transcripts during *in vitro* culture from MII oocyte stage until blastocyst stage. We used two different sources of media for embryo culture, commercially available COOK BVC/BVB and Menezo B2 [24]. The design of COOK media resides on the switch from pyruvate metabolite to glucose during post-compaction period. Menezo B2 represents the most complex medium used for cultivation from zygote till blastocyst stage.

#### 3.3.1 Expression of cullin 1 and cullin 1-like in bovine preimplantation embryo

Cullin 1-like (*CUL1*) transcript was more abundant at both 4-cell stage and 8-cell stage IVP embryos in our microarray study. There are five major categories of cullins in metazoan (*CUL1* through *CUL5*) and an additional vertebrate specific class containing *CUL7* and Parkin-like cytoplasmic protein (*PARC*) [25]. CUL1 forms ubiquitin ligase complexes SCF, which consist of three invariable components, Skp1, CUL1 (Cdc53 in yeast) and Rbx1, and a variable component F-box protein. SCF complexes mediate ubiquitination of proteins involved in cell-cycle progression, mainly during G1/S phase transition, and are also involved in regulation of centrosome duplication [26,27]. *Cul1* null mice embryos die around E5.5-E7.5 before the onset of gastrulation, showing signs of cyclin E dysregulation [28,29]. Attempts to derive *Cul1*$^{-/-}$ embryonic stem cells were unsuccessful, consistent with an essential role for Cul1 in proliferation of early embryonic cell types [29]. In RT-PCR for *CUL1* transcript, we have detected two products with the same length (data not shown) but different melting points (Fig. 2A), with the first product present from MII oocytes till early 8-cell stage embryos and the second product from late 8-cell stage embryos till blastocyst stage. The search of the Entrez Gene DNA database revealed that cullin 1-like and cullin 1 represent two different genes from cullin family (UniGene IDs **BT.36789**, resp. **BT.6490**), both on chromosome 4 but located in two different regions. Sequencing of amplified fragments confirmed the identity of the first fragment with Bos taurus cullin 1-like, transcript variant 1 mRNA (GeneBank ID **XM_589507.3**, 97-99% similarity from MII till 4-cell stage, 88% similarity for early 8-cell stage embryos) and the identity of the second fragment with Bos taurus cullin 1, transcript variant 3 mRNA (GeneBank ID **XM_876699**, 87% similarity for late 8-cell stage, 95-98% similarity from morula till hatched blastocyst) (Fig. 2B). To confirm the observed switch in these gene variants, we cultured IVP bovine embryos from 1-cell or 4-cell up to late 8-cell stage in the presence of α-amanitin, inhibitor of transcription by RNA polymerase II. Only low levels of cullin 1-like mRNA and no cullin 1 mRNA were detected in α-amanitin treated late 8-cell stage embryos, while cullin 1 mRNA was detected in control 8-cell stage embryos (Fig. 2A). CUL1 protein as detected by immunofluorescence staining was present throughout the whole

period of preimplantation development, with diffuse cytoplasmic localization (Fig. 2C) which is consistent with its function in protein ubiquitination and subsequent degradation [28,29]. Marin's [30] study of diversification of the cullin family in eukaryotes identified bovine cullin 1-like (RefSeq ID **XM_589507**) as orthologous to cullin 1 in other animal species. We have used Exalign tool [22] to compare exon-intron structure of both bovine *CUL1* genes to the genes in human, mouse and rat. This comparison showed that cullin 1 (**BT.6490**) shares the same structure (Supplementary File 5) with the human, mouse and rat cullin 1 genes and seems to be their true ortholog, while intronless cullin 1-like (**BT.36789**) probably emerged by duplication within the bovine genome. It remains to be solved whether both genes have similar function or not. Currently, there are 5 ESTs associated with cullin 1-like (**BT.36789**) in UniGene database, four of them belonging to the 2-cell IVP bovine embryo EST library (dbEST **15406**) and one belonging to the bovine oocyte cDNA SSH library (dbEST **17330**) [27]. Another 84 ESTs, representing many different tissues, including brain, liver, intestine, skin and muscle are associated with cullin 1 (**BT.6490**), corresponding to its ubiquitous expression and indispensible function in other species. Hwang et al. [31] detected by real-time RT-PCR with primers specific to cullin 1, transcript variant 3 mRNA expression of cullin 1 in bovine IVP morula, blastocyst and hatched blastocyst, but not in the 2-cell to 16-cell stage embryos. Together with our results, all this suggests that cullin 1-like, transcript variant 1 mRNA represents maternal transcript, which is gradually degraded after fertilization and cullin 1 transcript variant 3 represents new embryonic mRNA synthesized from 8-cell stage on. Similar developmental change has already been described in protein translation initiation factor *eIF-1A* gene during mouse embryonic genome activation [32]. The fact that a switch in transcript variants occurs around 8-cell stage, where major gene activation takes place and *CUL1* importance for cell cycle regulation, warrants its further investigation.

### 3.3.2 Expression of *FBL* transcript and localization of FBL protein in bovine IVP preimplantation embryo

Fibrillarin (*FBL*) was found to be more abundant in IVP than in IVD 4-cell stage embryos. FBL is localized to the fibrillar centres (FCs) and the dense fibrillar component (DFC) of nucleolus, where it is involved in primary rRNA transcript processing. Svarcova et al. [33] have shown recently that de-novo fibrillarin mRNA synthesis is required for re-formation of the functional nucleolus during the major genome activation period in cattle embryos. Previously, fibrillarin protein was first detected by immunofluorescence at late 8-cell stage in IVP bovine embryos, but was absent in embryos cultured in the presence of α-amanitin. In our study, qRT-PCR showed very low *FBL* mRNA level up to 4-cell stage, by early 8-cell stage levels of *FBL* transcript started to increase in both culture media, but this increase was much more pronounced in Menezo B2 medium as compared with Cook BVC/BVB medium (Fig. 4A). We have also studied FBL protein level and localization by immunofluorescence in *in vitro* matured oocytes and IVP embryos. Clear signal for FBL was detected in the GV stage oocyte nucleolus (Fig. 3, GV, arrowhead). From MII stage oocytes till 4-cell stage IVP embryos, FBL was undetectable. Contrary to the findings of [33], we have

detected the first FBL signal with pattern typical for newly forming nucleoli in early 8-cell stage IVP embryos in our culture conditions (Fig. 3, early 8-cell, arrowheads). This is similar to the results obtained in bovine embryos derived *in vivo* [34].

### 3.3.3 Expression profiling of *NOLC1*, *BUB3*, *PCAF*, *GABPA* and *CNOT4* during bovine preimplantation development

Transcript for other nucleolar protein, Nucleolar phosphoprotein p130, nucleolar and coiled-body phosphoprotein 1 (NOLC1, Nopp140) was found to be more abundant in IVP than in IVD 8-cell stage embryos in our microarray study. Phosphoprotein NOLC1 shuttles between nucleolus, cytoplasm and coiled bodies [35] and serves as chaperone for small nucleolar ribonucleoprotein particle (snoRNP) complexes. NOLC1 also functions as a transcription factor for RNA polymerase II [36] and interacts with casein kinase 2 [37] and RNA polymerase I [38]. The latter NOLC1 function suggests that it can be also involved in nucleologenesis during embryonic development and in maintenance of nucleolus integrity [39]. In this study, qRT-PCR profile of *NOLC1* transcript in IVP oocytes and embryos showed low transcript levels in 2-cell stage and 4-cell stage embryos and increasing transcript levels from early 8-cell stage till blastocyst. In both *in vitro* culture conditions, *NOLC1* transcript levels were higher at both 4-cell stage and 8-cell stage than in IVD embryos, confirming results from microarrays (Fig. 4B).

Spindle checkpoint component BUB3 is essential for establishment of microtubule-kinetochore attachment during mitosis [40] and its deletion on mouse model results in early embryonic lethality around day E6.5-E7.4 accompanied by an accumulation of mitotic errors [41]. *BUB3* transcript was identified in two microarray studies as up-regulated in bovine and human oocytes [11,42], underlining its importance for correct preimplantation embryo cell cycle progression. We have identified *BUB3* transcript as more abundant in 8-cell stage IVP embryos, which may indicate requirements for tighter checkpoint controls in non-optimal *in vitro* conditions. qRT-PCR profiling of *BUB3* level during *in vitro* development showed a slow decrease in transcript levels from oocytes to early 8-cell stage and then increase in morula and blastocyst stage in both *in vitro* culture conditions (Fig. 4C).

The p130/CBP associated-factor (PCAF) is a protein with intrinsic histone acetylase activity [43], which participates in transcriptional activation by interaction with transcription factors and by chromatin remodelling. While null mutation of *PCAF* is not lethal, probably because of the compensating effect of closely related PCAF-B/GCN5 protein [44], composite *PCAF/GCN5* mutants show even earlier embryonic lethality than *GCN5* mutation alone, confirming the role of PCAF in regulation of early embryonic transcription. In our microarray experiment, *PCAF* transcript was more abundant in 8-cell stage IVP embryos and qRT-PCR profile confirmed this result, showing very low transcript levels up to 4-cell stage, with small increase in early 8-cell stage followed by steeper increase from late 8-cell to blastocyst stage and decrease in hatched blastocyst stage (Fig. 4D).

DNA binding protein GABPA is a component of ETS transcription complex GABP (also known as NRF-2 in humans). This transcription factor is essential for regulation

of expression of mitochondrial respiration chain genes and its deletion results in embryo lethality at preimplantation stage [45]. Kinoshita et al. [46] showed that Gabpa is involved also in regulation of Oct4 level in the mouse embryonic stem cell lines, probably by down-regulation of Oct4 repressors. As Oct4 is necessary for self-renewal of embryonic stem cells *in vitro* and formation of inner cell mass *in vivo*, this Gabpa function also explains the early lethality of null phenotype. Again we have found *GABPA* to be more abundant in 8-cell stage IVP embryos relative to their IVD counterparts. The expression profiling of IVP embryos by qRT-PCR showed moderate *GABPA* transcript levels from MII oocytes to 4-cell stage embryos, with significantly increased level from early 8-cell stage to blastocyst (Fig. 4E). This is consistent with the requirement of this transcription factor for OCT4 level regulation at the time of blastocyst formation.

CNOT4 protein is a component of a CCR4-NOT multifunctional complex. CCR4-NOT is composed of at least 9 identified subunits and is involved in the regulation of transcription by controlling the distribution of TFIID transcription factor across promoters [47], RNA deadenylation and both cytoplasmic [48] and nuclear RNA degradation [49], transcription coupled DNA repair [50] and protein ubiquitination [51]. CNOT4 is an E3 ubiquitin ligase, capable of direct interactions with UbcH6, UbcH9 and UbcH5B E2 ubiquitin conjugating enzymes [51]. The first known substrate of CNOT4 ubiquitin ligase activity was an EGD/NAC [52], nascent polypeptide-associated complex, functioning as a chaperone for newly synthesized proteins and also as transcriptional coactivator. CNOT4 also directly regulates by ubiquitination the protein level of both yeast and human histone demethylase Jhd2 (resp. JARID1C) and in this way it globally regulates transcription [53]. There is currently little information about the involvement of CCR4-NOT complex and CNOT4 subunit particularly in the early mammalian development, but its many different functions and conservation of its expression throughout eukaryotes suggest its importance. In our study, *CNOT4* transcript levels were constantly decreasing from 2-cell stage embryos till morula stage and slightly increasing thereafter. *CNOT4* was identified as a transcript more abundant in the IVP than in IVD 4-cell stage embryos on microarray, but this difference was not confirmed by qRT-PCR, while at the late 8-cell stage we have detected significantly more transcript in IVD embryos, compared to IVP (Fig. 4F).

We used two different cultivation media (Menezo B2, COOK) for in vitro cultivation. The differences in gene expression were minimal between embryos cultivated in Menezo B2 or COOK during development from 2-cell to morula stage. Embryos cultivated in Menezo B2 medium exhibited higher level of mRNA in all investigated genes at blastocyst and hatched blastocyst. This difference was not significant in majority of investigated genes because of high variability of mRNA level in blastocyst and hatched blastocyst stages. Most probably, embryos in Menezo B2 medium developed faster in blastocyst stage and resulted higher number of cells in individual blastocysts exhibited higher level of mRNA. From this point of view, Menezo B2 medium is superior to COOK medium for the production of bovine blastocysts.

### 3.4 Conclusions

In summary, using microarray analysis and protein-protein interaction modelling, *CUL1, FBL* and *CNOT4* genes were chosen for subsequent particular analysis. The transcription variant 1 of Cullin 1-like mRNA was present from MII oocyte till early 8-cell stage, the transcription variant 3 of Cullin 1 became prevalent from late 8-cell stage onward. Cullin 1-like, transcript variant 1 mRNA represents maternal transcript, which is gradually degraded after fertilization and Cullin 1 transcript variant 3 represents new embryonic mRNA synthesized from 8-cell stage on. The molecular basis for this change in Cullin gene expression during bovine embryonic genome activation is not known.

New fibrillarin protein was detected by immunofluorescence already in early 8-cell stage, and its detection correlated with increased level of fibrillarin RNA.

The qRT-PCR analysis revealed significant differences in the level of *BUB3*, *NOLC1*, *PCAF*, *GABPA* and *CNOT4* gene transcripts between IVD and IVP embryos in late 8-cell stage. The complex of these genes represents a suitable tool for answering questions concerning normal IVD embryos development and optimization of IVP embryo culture conditions.

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## References

[1] Lonergan P, Fair T. In vitro-produced bovine embryos: dealing with the warts. *Theriogenology* 2008**;** 69: 17-22.

[2] Mourot M, Dufort I, Gravel C, Algriany O, Dieleman S, Sirard MA. The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels. *Mol Reprod Dev* 2006**;** 73: 1367-79.

[3] Russell DF, Baqir S, Bordignon J, Betts DH. The impact of oocyte maturation media on early bovine embryonic development. *Mol Reprod Dev* 2006**;** 73: 1255-70.

[4] Watson AJ, De Sousa P, Caveney A, Barcroft LC, Natale D, Urquhart J, Westhusin ME. Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. *Biol Reprod* 2000**;** 62: 355-64.

[5] Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev* 1990**;**26:90-100.

[6] Memili E, First NL. Control of gene expression at the onset of bovine embryonic development. *Biol Reprod* 1999**;** 61: 1198-207.

[7] Memili E, Dominko T, First NL. Onset of transcription in bovine oocytes and preimplantation embryos. *Mol Reprod Dev* 1998**;** 51: 36-41.

[8] Gutierrez-Adan A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, Boland MP, Lonergan P. Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Mol Reprod Dev* 2004**;** 68: 441-8.

[9] McHughes CE, Springer GK, Spate LD, Li R, Woods R, Green MP, Korte SW, Murphy CN, Green JA, Prather RS. Identification and quantification of differentially represented transcripts in in vitro and in vivo derived preimplantation bovine embryos. *Mol Reprod Dev* 2009**;**76:48-60.

[10] Corcoran D, Fair T, Park S, Rizos D, Patel OV, Smith GW, Coussens PM, Ireland JJ, Boland MP, Evans AC , Lonergan P. Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos. *Reproduction* 2006**;** 131: 651-60.

[11] Adjaye J, Herwig R, Brink TC, Herrmann D, Greber B, Sudheer S, Groth D, Carnwath JW, Lehrach H, Niemann H. Conserved molecular portraits of bovine

and human blastocysts as a consequence of the transition from maternal to embryonic control of gene expression. *Physiol Genomics* 2007 **;** 31: 315-27.

[12] Vigneault C, Gravel C, Vallee M, McGraw S, Sirard MA. Unveiling the bovine embryo transcriptome during the maternal to embryonic transition. *Reproduction* 2008.

[13] Sirard MA, Dufort I, Vallee M, Massicotte L, Gravel C, Reghenas H, Watson AJ, King WA, Robert C. Potential and limitations of bovine-specific arrays for the analysis of mRNA levels in early development: preliminary analysis using a bovine embryonic array. *Reprod Fertil Dev* 2005**;** 17: 47-57.

[14] Pavlok A, Lucas-Hahn A, Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol Reprod Dev* 1992**;** 31: 63-7.

[15] Kues WA, Sudheer S, Herrmann D, Carnwath JW, Havlicek V, Besenfelder U, Lehrach H, Adjaye J, Niemann H. Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo. *Proc Natl Acad Sci USA* 2008**;** 105: 19768-73.

[16] Besenfelder U, Havlicek V, Mosslacher G, Brem G. Collection of tubal stage bovine embryos by means of endoscopy. A technique report. *Theriogenology* 2001**;** 55: 837-45.

[17] Robertson, I., Nelson, R. E., Certification and identification of the embryo. In: Manual of the International Embryo Transfer Society: a procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures, 3rd ed.; 1998, pp. 103-116.

[18] Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 2003**;** 34: 374-8.

[19] Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* 2002**;** 30: e15.

[20] Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci* 2001**;** 98: 5116-21.

[21] Brown KR, Jurisica I. Online predicted human interaction database. *Bioinformatics* 2005**;** 21: 2076-82.

[22] Pavesi G, Zambelli F, Caggese C, Pesole G. Exalign: a new method for comparative analysis of exon-intron gene structures. *Nucleic Acids Res* 2008**;** 36: e47.

[23] Bui LC, Leandri RD, Renard JP, Duranthon V. SSH adequacy to preimplantation mammalian development: scarce specific transcripts cloning despite irregular normalisation. *BMC Genomics* 2005**;** 6: 155.

[24] Staessen C, Janssenswillen C, De Clerck E, Van Steirteghem A. Controlled comparison of commercial media for human in-vitro fertilization: Menezo B2 medium versus Medi-Cult universal and BM1 medium. *Hum Reprod* 1998**;** 13: 2548-54.

[25] Bosu DR, Kipreos ET. Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell Div* 2008**;** 3: 7.

[26] Freed E, Lacey KR, Huie P, Lyapina SA, Deshaies RJ, Stearns T, Jackson PK. Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. *Genes Dev* 1999**;** 13: 2242-57.

[27] Pennetier S, Uzbekova S, Guyader-Joly C, Humblot P, Mermillod P, Dalbies-Tran R. Genes preferentially expressed in bovine oocytes revealed by subtractive and suppressive hybridization. *Biol Reprod* 2005**;** 73: 713-20.

[28] Dealy MJ, Nguyen KV, Lo J, Gstaiger M, Krek W, Elson D, Arbeit J, Kipreos ET, Johnson RS. Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. *Nat Genet* 1999**;** 23: 245-8.

[29] Wang Y, Penfold S, Tang X, Hattori N, Riley P, Harper JW, Cross JC, Tyers M. Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. *Curr Biol* 1999**;** 9: 1191-4.

[30] Marin I. Diversification of the cullin family. *BMC Evol Biol* 2009**;** 9: 267.

[31] Hwang KC, Cui XS, Park SP, Shin MR, Park SY, Kim EY, Kim NH. Identification of differentially regulated genes in bovine blastocysts using an annealing control primer system. *Mol Reprod Dev* 2004**;** 69: 43-51.

[32] Davis W Jr, Schultz RM. Developmental change in TATA-box utilization during preimplantation mouse development. *Dev Biol* 2000**;** 218: 275-83.

[33] Svarcova O, Laurincik J, Avery B, Mlyncek M, Niemann H, Maddox-Hyttel P. Nucleolar development and allocation of key nucleolar proteins require de novo transcription in bovine embryos. *Mol Reprod Dev* 2007**;** 74: 1428-35.

[34] Laurincik J, Schmoll F, Mahabir E, Schneider H, Stojkovic M, Zakhartchenko V, Prelle K, Hendrixen PJ, Voss PL, Moeszlacher GG, Avery B, Dieleman SJ, Besenfelder U, Muller M, Ochs RL, Wolf E, Schellander K, Maddox-Hyttel P. Nucleolar proteins and ultrastructure in bovine in vivo developed, in vitro produced, and parthenogenetic cleavage-stage embryos. *Mol Reprod Dev* 2003**;** 65: 73-85.

[35] Isaac C, Yang Y, Meier UT. Nopp140 functions as a molecular link between the nucleolus and the coiled bodies. *J Cell Biol* 1998**;** 142: 319-29.

[36] Miau LH, Chang CJ, Tsai WH, Lee SC. Identification and characterization of a nucleolar phosphoprotein, Nopp140, as a transcription factor. *Mol Cell Biol* 1997**;** 17: 230-9.

[37] Li D, Meier UT, Dobrowolska G, Krebs EG. Specific interaction between casein kinase 2 and the nucleolar protein Nopp140. *J Biol Chem* 1997**;** 272: 3773-9.

[38] Chen HK, Pai CY, Huang JY, Yeh NH. Human Nopp140, which interacts with RNA polymerase I: implications for rRNA gene transcription and nucleolar structural organization. *Mol Cell Biol* 1999**;** 19: 8536-46.

[39] Baran V, Brochard V, Renard JP, Flechon JE. Nopp 140 involvement in nucleologenesis of mouse preimplantation embryos. *Mol Reprod Dev* 2001**;** 59: 277-84.

[40] Logarinho E, Resende T, Torres C, Bousbaa H. The human spindle assembly checkpoint protein Bub3 is required for the establishment of efficient kinetochore-microtubule attachments. *Mol Biol Cell* 2008**;** 19: 1798-813.

[41] Kalitsis P, Earle E, Fowler KJ, Choo KH. Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev* 2000**;** 14: 2277-82.

[42] Gasca S, Pellestor F, Assou S, Loup V, Anahory T, Dechaud H, De Vos J, Hamamah S. Identifying new human oocyte marker genes:

a microarray approach. *Reprod Biomed Online* 2007**;** 14: 175-83.

[43] Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* 1996**;** 382: 319-24.

[44] Yamauchi T, Yamauchi J, Kuwata T, Tamura T, Yamashita T, Bae N, Westphal H, Ozato K, Nakatani Y. Distinct but overlapping roles of histone acetylase PCAF and of the closely related PCAF-B/GCN5 in mouse embryogenesis. *Proc Natl Acad Sci* 2000**;** 97: 11303-6.

[45] Ristevski S, O'Leary DA, Thornell AP, Owen MJ, Kola I, Hertzog PJ. The ETS transcription factor GABPalpha is essential for early embryogenesis. *Mol Cell Biol* 2004**;** 24: 5844-9.

[46] Kinoshita K, Ura H, Akagi T, Usuda M, Koide H, Yokota T. GABPalpha regulates Oct-3/4 expression in mouse embryonic stem cells. *Biochem Biophys Res Commun* 2007**;**353:686-91.

[47] Lenssen E, James N, Pedruzzi I, Dubouloz F, Cameroni E, Bisig R, Maillet L, Werner M, Roosen J, Petrovic K, Winderickx J, Collart MA, De Virgilio C. The Ccr4-Not complex independently controls both Msn2-dependent transcriptional activation--via a newly identified Glc7/Bud14 type I protein phosphatase module--and TFIID promoter distribution. *Mol Cell Biol* 2005**;** 25: 488-98.

[48] Tucker M, Valencia-Sanchez MA, Staples RR, Chen J, Denis CL, Parker R. The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae. *Cell* 2001**;** 104: 377-86.

[49] Azzouz N, Panasenko OO, Colau G, Collart MA. The CCR4-NOT complex physically and functionally interacts with TRAMP and the nuclear exosome. *PLoS One* 2009**;** 4: e6760.

[50] Gaillard H, Tous C, Botet J, Gonzalez-Aguilera C, Quintero MJ, Viladevall L, Garcia-Rubio ML, Rodriguez-Gil A, Marin A, Arino J, Revuelta JL, Chavez S, Aguilera A. Genome-wide analysis of factors affecting transcription elongation and DNA repair: a new role for PAF and Ccr4-not in transcription-coupled repair. *PLoS Genet* 2009**;** 5: e1000364.

[51] Albert TK, Hanzawa H, Legtenberg YI, de Ruwe MJ, van den Heuvel FA, Collart MA, Boelens R, Timmers HT. Identification of a ubiquitin-protein ligase subunit within the CCR4-NOT transcription repressor complex. *EMBO J* 2002**;** 21: 355-64.

[52] Panasenko O, Landrieux E, Feuermann M, Finka A, Paquet N, Collart MA. The yeast Ccr4-Not complex controls ubiquitination of the nascent-

associated polypeptide (NAC-EGD) complex. *J Biol Chem* 2006**;** 281: 31389-98.

[53] Mersman DP, Du HN, Fingerman IM, South PF, Briggs SD. Polyubiquitination of the demethylase Jhd2 controls histone methylation and gene expression. *Genes Dev* 2009**;** 23: 951-62.

## Table 1

**Primer sequences and PCR conditions used to evaluate expression of selected genes in bovine embryos by qRT-PCR.**

| Official symbol (Gene) | GenBank ID | Primer sequence (5'->3') | Amplicon size (bp) | Annealing temperature (°C) |
|---|---|---|---|---|
| ***BUB3*** | XM_879565 | Forward - CAGGGTTATGTATTAAGTTCTATC<br>Reverse - TCTGTGACACTTGAAGGC | 102 | 50 |
| ***CUL1*** | XM_875152 | Forward – CTGAAGTTCTATACTCAACAATG<br>Reverse - ACAATCTCTCCAAGTCACC | 162 | 50 |
| ***NOLC1*** | XM_590941<br>NM_001075608 | Forward - GAGCGAGCCAATCAGGTTC<br>Reverse - AGAGTTGACTTGGACAGAGATG | 114 | 55 |
| ***FBL*** | XM_581057 | Forward – AAGCGGACCAACATTATTC<br>Reverse - GCATTCAGGGCTACAATC | 134 | 50 |
| ***PCAF*** | XM_613744 | Forward - ATATACTCTGCCCAACTGATAATG<br>Reverse - CAAGACAGGTAAGGTGTATGATG | 179 | 55 |
| ***GABPA*** | AF057717 | Forward - TGACTGATATACACCTCACTACAC<br>Reverse - ATCTCATTCATCTGTTGTTCTTGG | 166 | 55 |
| ***CNOT4*** | NM_001035432 | Forward – ACTCGTTCAGTGGTCTCTC<br>Reverse - GGTCTTCCTTTGCGTCAG | 199 | 55 |

***BUB3*** (Budding uninhibited by benzimidazoles), ***CUL1*** (Cullin 1), ***NOLC1*** (Nucleolar and coiled-body phosphoprotein 1, nucleolar phosphoprotein p130 – NOPP140), ***FBL*** (Fibrillarin), ***PCAF*** (p300/CBP-associated factor), ***GABPA*** (GA-binding protein transcription factor, alpha subunit), ***CNOT4*** (CCR4-NOT transcription complex, subunit 4)

## Figure legends:

Fig. 1: Simplified view of the network of possible protein-protein interactions, generated by querying I2D database for candidate genes identified on microarray as differentially regulated between IVD and IVP embryos. Nodes representing candidate gene protein orthologs are shown as circles and triangles with protein name next to node and genes selected for qRT-PCR verification are highlighted by increased node size with label centred over the node. Colour of nodes represents Gene Ontology function. Four clusters of highly interconnected nodes (cliques) identified within the network are highlighted by coloured edges, biggest group (sorted by max. degree) containing FBL in dark blue, second biggest group containing CUL1 in red, the third group containing CNOT4 in green and last group in light blue.

Fig. 2: Switch from expression of *CUL1-like* transcript variant 1 to *CUL1* transcript variant 3 during preimplantation embryo development. A) Melting curves show the presence of two different products obtained by RT-PCR (primers designed against *CUL1* transcript variant 2, cDNA present on microarray) from IVP (COOK BVC/BVB and Menezo B2 culture systems) and IVD embryos *(left column)*. More detailed study showed an earlier switch in transcript variants in COOK BVC/BVB culture system (around 60 hpf) compared to Menezo B2 medium (around 66 hpf). When α-amanitin was added to the culture medium at a final concentration of 100 μg/ml (from late 1- to 8-cell stage or from 4- to 8-cell stage), only the product with lower melting temperature was detected in late 8-cell stage embryos, while product with higher melting temperature was detected in control late 8-cell stage embryos *(right column)*. B) Sequencing of RT-PCR products. Sequences detected in IVP MII oocytes, 2-cell, 4-cell, early 8-cell and α-amanitin treated late 8-cell stage IVP embryos and 2-cell stage, 4-cell stage and 8-cell stage IVD embryos cluster together with *CUL1-like* transcript variant 1, while sequences detected in IVP late 8-cell stage embryos, morulas, blastocysts and hatched blastocysts and 8-16-cell stage IVD embryos cluster together with *CUL1* transcript variant 3. C) Immunofluorescence staining of CUL1 in IVP oocytes and embryos shows protein presence throughout preimplantation development. All scale bars represent 20 μm.

Fig. 3: Fibrillarin protein localization in bovine oocytes and IVP embryos. GV) FBL signal was detected in nucleolus of GV stage oocyte (arrowhead); MII) while clear signal could be detected in nucleoli of cumulus cells (arrows), no localized FBL signal was detected in MII stage oocyte (arrowhead shows metaphase II chromosomes); 2-cell) no localized FBL signal was detected; 4-cell) no FBL signal was present in nuclei of embryo (arrowheads), while clear signal could be detected in nucleoli of cumulus cells (arrows); early 8-cell) faint but clearly localized FBL signal was detected in nuclei of early 8-cell stage embryo, showing formation of new nucleoli (arrowheads); late 8-cell, 16-cell, morula) stronger localized FBL signal was detected (arrowheads). All scale bars represent 20 μm.

Fig. 4: Relative abundance of *FBL*, *NOLC1*, *BUB3*, *PCAF*, *GABPA* and *CNOT4* mRNAs in bovine IVP (oo, MII oocyte; 2c, 2-cell; 4c, 4-cell; e8c, early 8-cell; l8c, late 8-cell stage; bl., blastocyst; h. bl., hatched blastocyst) and IVD embryos (2c, 2-cell; 4c, 4-cell; l8c, late 8-cell stage) was estimated by one-step RT-PCR with real time detection. The results were normalized according to the relative concentration of the external standard (Luciferase mRNA, 1 pg per oocyte/embryo) and represent mean ± SD from 3 independent qRT-PCR experiments (3 different pools of oocytes/embryos). $^{a-e}$Same superscripts above the columns indicate significant differences ($P<0.05$), asterisks indicate significance at $P<0.01$.

Supplementary File 1: Excel table of cDNA clones from 3 SSH libraries generated from bovine oocytes and embryos. Lib.30 (4C-MII): IVP 4-cell stage embryos subtracted from *in vitro* matured MII stage oocytes, Lib.31 (4C - 8C): IVP 8-cell stage embryos subtracted from IVP 4-cell stage embryos and Lib.32 (8C - 4C): IVP 4-cell stage embryos were subtracted from IVP 8-cell stage embryos. cDNAs labelled in green were included in the BlueChip, V3 array design.

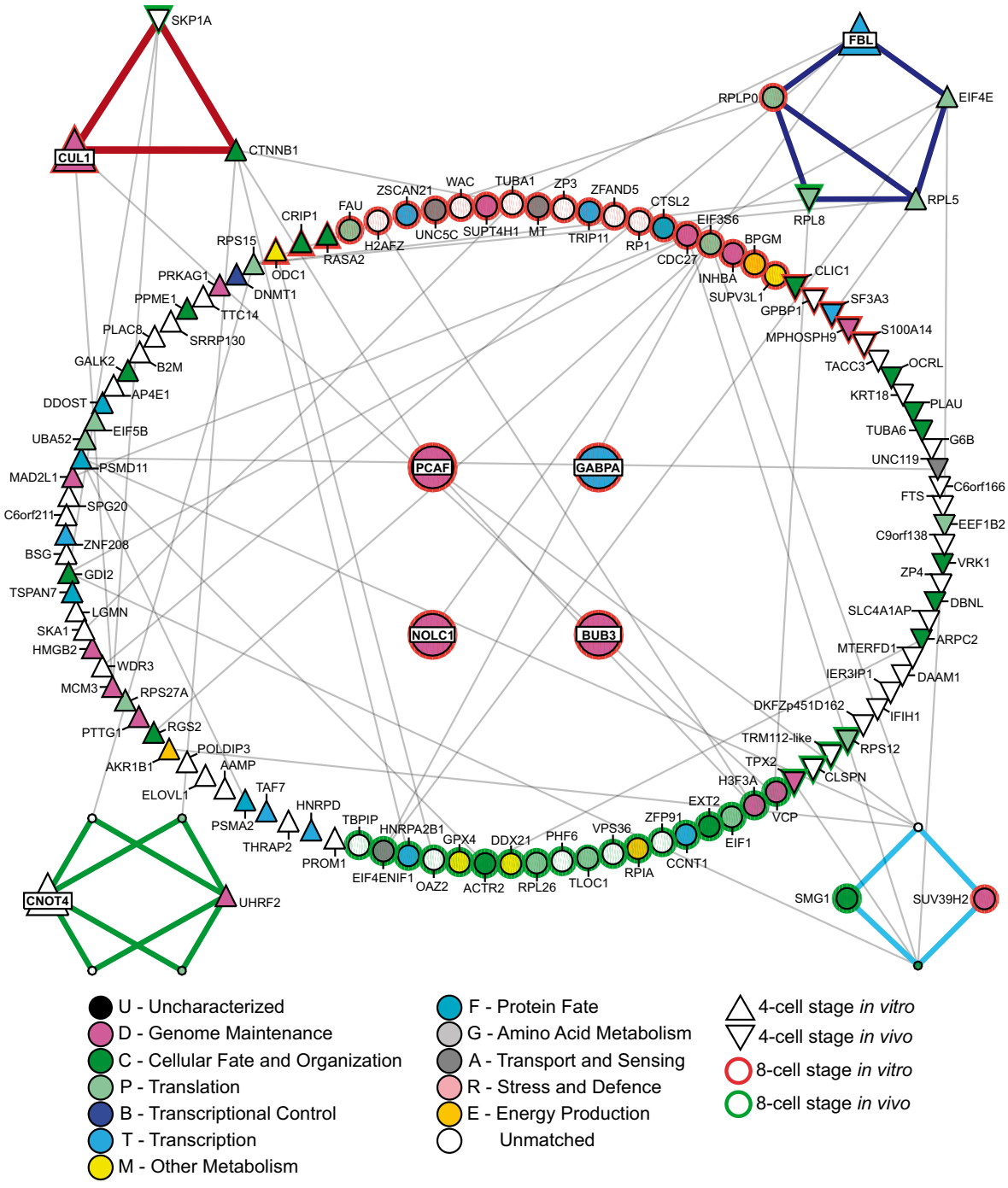
Supplementary File 2: Excel table of candidate genes identified as differentially expressed by Significance analysis of microarrays (SAM), with Protein/Swiss-Prot accession numbers of their human orthologs, used for protein-protein interaction network modelling.

Supplementary File 3: List of possible protein interaction partners of candidate genes, as identified using I2D database, version 1.8.

Supplementary File 4: Protein-protein interaction network file, corresponding to Fig.1. The data are in NAViGaTOR 2.0 xml format and can be opened in any NAViGaTOR 2 version (http://ophid.utoronto.ca/navigator/).

Supplementary File 5: Exon-intron structure of bovine cullin 1 gene (UniGene **BT.6490**, Query: RefSeq ID **XM_876699**) in comparison with human, mouse and rat cullin 1 gene. Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition.

Fig. 1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CTSL2
FAU
CRIP1
UNC5C
SUPT4H1
MT
TRIP11
RP1
EIF3S6
H2AFZ
RASA2
CDC27
BPGM
RPS15
ODC1
INHBA
CLIC1
PRKAG1
DNMT1
SUPV3L1
SF3A3
PPME1
TTC14
GPBP1
S100A14
PLAC8
SRRP130
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GALK2
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EIF5B
TUBA6
G6B
PCAF
GABPA
MAD2L1
PSMD11
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C6orf211
SPG20
FTS
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BSG
ZNF208
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TSPAN7
GDI2
ZP4
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LGMN
NOLC1
BUB3
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VCP
THRAP2
TBPIP
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EIF1
PROM1
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GPX4
DDX21
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ACTR2
RPL26
TLOC1
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CNOT4
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SMG1
SUV39H2
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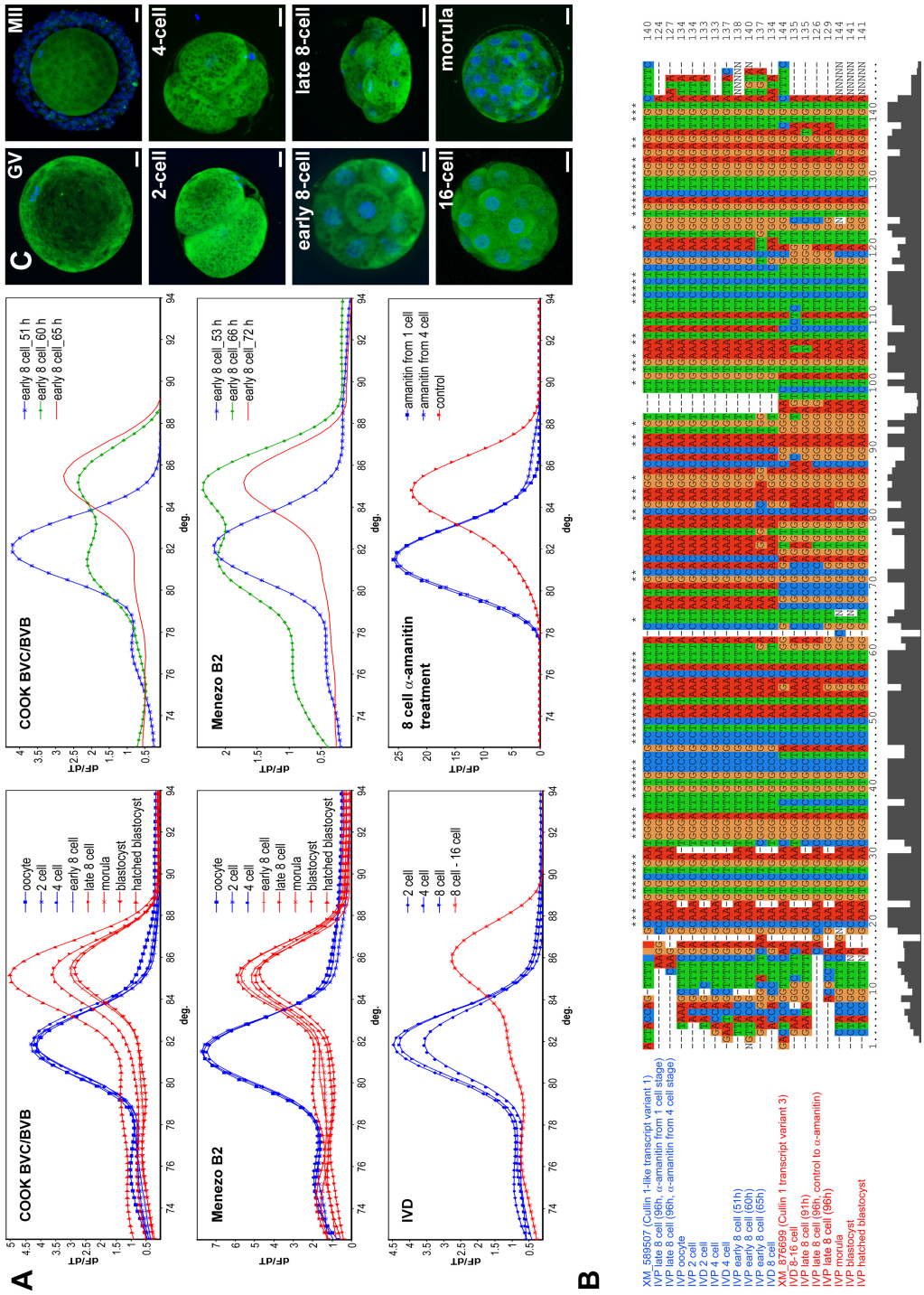
D - Genome Maintenance
C - Cellular Fate and Organization
P - Translation
B - Transcriptional Control
T - Transcription
M - Other Metabolism
F - Protein Fate
G - Amino Acid Metabolism
A - Transport and Sensing
R - Stress and Defence
E - Energy Production
Unmatched
4-cell stage in vitro
4-cell stage in vivo
8-cell stage in vitro
8-cell stage in vivo

Fig. 2


A
COOK BVC/BVB
Menezo B2
IVD
8 cell α-amanitin treatment
oocyte
2 cell
4 cell
early 8 cell
late 8 cell
morula
blastocyst
hatched blastocyst
2 cell
4 cell
8 cell
8 cell - 16 cell
early 8 cell_51 h
early 8 cell_60 h
early 8 cell_65 h
early 8 cell_53 h
early 8 cell_66 h
early 8 cell_72 h
amanitin from 1 cell
amanitin from 4 cell
control
dF/dT
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GV
MII
2-cell
4-cell
early 8-cell
late 8-cell
16-cell
morula

Fig. 3

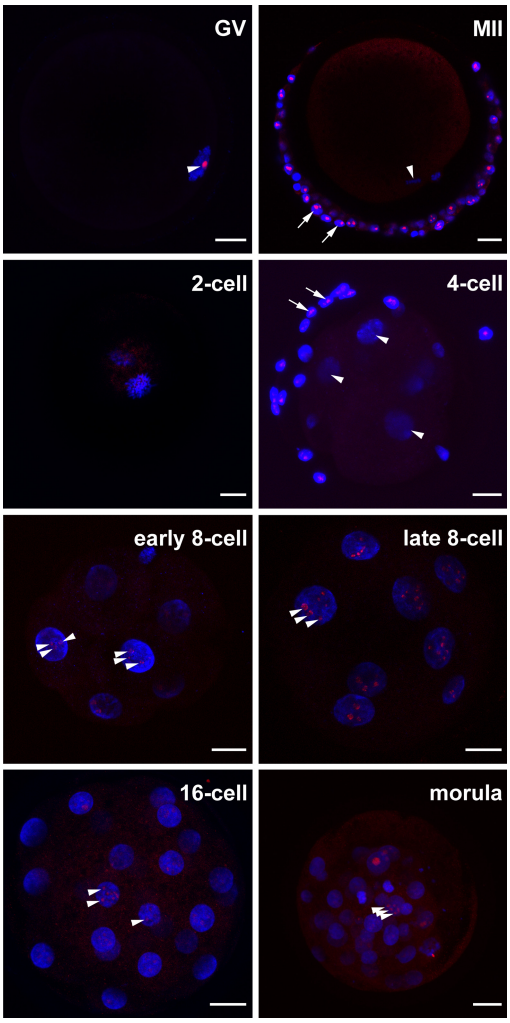
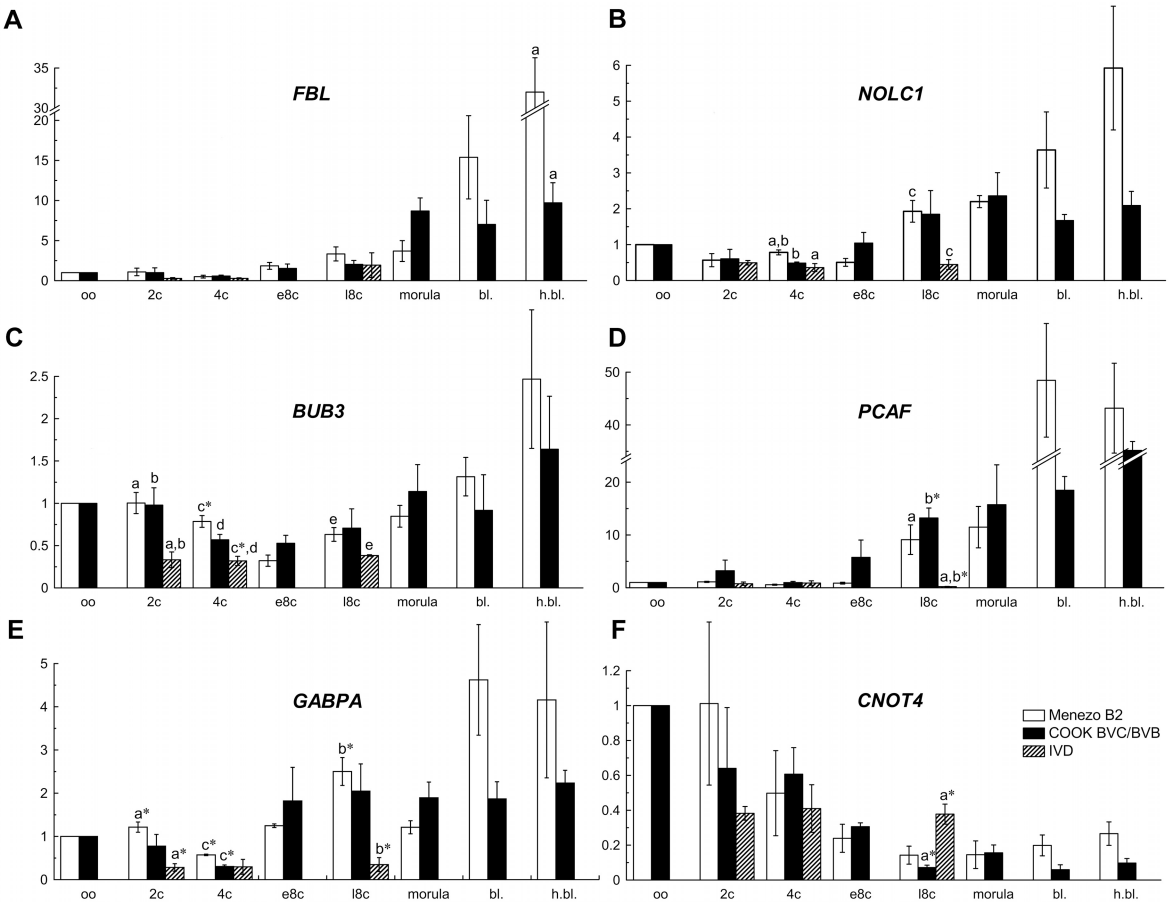


Fig. 4


A
FBL
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l8c
morula
bl.
h.bl.
Menezo B2
COOK BVC/BVB
IVD

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*Short communication*

# ULTRASTRUCTURAL ANALYSIS OF BOVINE SOMATIC CELL NUCLEAR TRANSFER (SCNT) EMBRYOS DURING THE FIRST CELL CYCLE

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## ABSTRACT

At somatic cell nuclear transfer (SCNT), the differentiated somatic cell genome is deprogrammed into a totipotent state of expression by unknown factors in the oocyte. The immediate events of this deprogramming are poorly understood and this study was designed in order to evaluate the global transcription and morphology of this event. Bovine SCNT embryos were produced from starved bovine fibroblasts and fixed at 0.5, 1, 2, 3, 4, 8, 12 and 16 h post-activation (hpa). The SCNT embryos were processed for autoradiography following $^{3}$H–uridine incubation and transmission electron microscopy. Likewise, starved and non-starved fibroblasts were processed for autoradiography and TEM. Fibroblasts displayed strong transcriptional activity and active fibrillo-granular nucleoli. None of the reconstructed embryos, however, displayed transcriptional activity. SCNT embryos fixed at 0.5 hpa displayed condensed chromatin invested only in a partial nuclear envelope. Abundant population of somatic cell mitochondria was concentrated around the nuclei at 0.5 to 4 hpa. At 1-3 hpa, a chromatin decondensation and nuclear envelope formation were observed. In all SCNT embryos at 4, 8, 12, and 16 hpa, a complete nuclear envelope surrounding large pronucleus-like nucleus with abundant euchromatic and sparse heterochromatic areas was observed. The first nucleolus-related structures in SCNT embryos were observed at 1.5 hpa and only in the nuclei with a complete nuclear envelope. At 1.5-4 hpa, the nucleolus-related structures appeared either as bodies including large fibrillar centres and a granular component, but no a dense fibrillar component, or as electron-dense spheres, i.e. nucleolus precursor bodies (NPBs). Since 4 hpa, the somatic cell nucleus gained a PN-like appearance and displayed NPBs suggesting an ooplasmic control of development.

**Keywords:** SCNT; bovine embryo; first cell cycle; nucleus, nucleolus; ultrastructure

## INTRODUCTION

There are two basic strategies for the cloning of mammals by SCNT that are able to produce embryos capable of development to term. Both these methods require the removal of the nuclear material from the oocyte and differ only in the way in which the nuclear material of the donor cell is introduced and the subsequent activation of the reconstructed embryos (Armstrong, 2006).

Application of MII-arrested non-activated oocytes is a far more effective for supporting development of the embryos reconstructed with differentiated nuclei than are activated oocytes. Therefore, a success in chromatin remodelling, from differentiated nuclei to the totipotent ones depends on the cell cycle stage of the recipient's cytoplasm. The ability to allow remodelling of chromatin apparently exists in MII-arrested non-activated oocytes and disappears after activation (Kim et al., 2002).

Of prime importance in these events is a cytoplazmatic activity termed maturation /meiosis/ mitosis-promoting factor (MPF) (Campbell et al., 1996). All nuclei that are transferred into the cytoplast with a high

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MPF activity undergo structural and functional remodelling and thus adopt the of behavioural pattern characteristic for the native nucleus. Structural reorganization of transferred nuclei (chromatin condensation/decondesation, nuclear envelope breakdown NEBD/ reformation, reorganization of nucleoli, nuclear swelling) seems to be a prerequisite of undertaking new functions in the heterologous cytoplasm (DNA synthesis, RNA synthesis) (Szollosi et al., 1988; Campbell et al., 1996).

In animal cloning, the highly differentiated donor nucleus must be properly dedifferentiated, cease its own program of gene expression, and express genes required for early embryo development. (Chen et al., 2006). Reprogramming involves a series of molecular events controlling gene expression, and has a profound effect on the nuclear architecture. Evidently the gene expression is silenced during the initial phases of reprogramming, which can be monitored by disassembly of the nucleolus (Misteli et al., 2003). Reprogramming of the donor nucleus is, among other features, indicated by a synthesis of ribosomal RNA (rRNA). The initiation of rRNA synthesis is simultaneously reflected in the nuclear morphology as a transformation of the nucleolus precursor body into a functional rRNA synthesizing nucleolus with a characteristic ultrastructure: fibrillar centres house the enzymatic apparatus for the transcriptional process, the dense fibrillar component carries the primary unprocessed transcript, and the granular component represent processed transcripts associated with proteins in the form of preribosomal particles (Wachtler and Stahl, 1993). This would suggest that the nucleolar morphology can be used as a marker for RNA synthesis and reprogramming of the donor nucleus in the nuclear transfer-derived embryos (Kanka et al., 1999).

However, no detailed investigations have been carried out on the ultrastructure and transcriptional activity during early genomic deprogramming i.e. first cell cycle in bovine embryos reconstructed by nuclear transfer from bovine fibroblasts to non-activated cytoplast. The objective of this study was to investigate morphological changes and RNA synthesis in the nuclei from cultured starved bovine fibroblast, when introduced into the enucleated metaphase II oocytes.

## MATERIAL AND METHODS

### Oocyte recovery and *in vitro* maturation

*In vitro* production of bovine embryos was performed using established protocols (Wrenzycki et al., 2002).

### Somatic cell nuclear transfer (SCNT)

Adult female fibroblasts used for the nuclear transfer procedure were established from ear skin samples collected from an abattoir. The tissue was cut and dispersed in 0.1% trypsin solution. The cell suspension was maintained in DMEM-F12. At 90% confluence the cell monolayer was trypsinized and harvested cells were adjusted to the concentration of a 1 million cells/ml and then either frozen in 10% DMSO or returned to the culture. The fibroblasts were induced to enter a period of quiescence (presumptive $G_0$) by a serum starvation for 3 days (0.5% FCS). Immediately before transfer into an enucleated oocyte, a suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted, resuspended and maintained in TCM-air medium.

For the production of SCNT embryos, metaphase II oocytes were placed into a TCM-air medium containing 5 µg/ml Hoechst 33342 and 7.5 µg/ml cytochalasin B for 8 min. Oocytes were enucleated by aspirating the first polar body and the metaphase II plate. Single fibroblast was transferred into the perivitelline space of the recipient oocyte using a 30µm pipette. Oocyte-fibroblast cell couplets were electrically fused by 1-2 DC pulses of 0.7 kV for 30 µsec in a 0.285 M mannitol based medium containing 0.1mM $MgSO_4$ and 0.05% BSA with an Eppendorf Multiporator machine (Hamburg, Germany). Fused cell hybrids were chemically activated by 5 µM ionomycin (Sigma) for 5 min followed by a 3-4 h incubation in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma). After activation, the embryos were washed and cultured in 30 µl droplets of SOFaa medium supplemented with 0.4% BSA at 39°C in 5% $O_2$, 5% $CO_2$ and 90% $N_2$ in Modular incubator chambers (ICN Biomedicals, Inc., Aurora, No. 615300, Ohio, USA). The embryos were collected at 0.5 hours post activation (hpa); 1 hpa; 1.5 hpa; 2 hpa; 3 hpa; 4 hpa; 8 hpa; 12 hpa and 16 hpa.

### $^3$H-Uridine Incubation for Autoradiography

Embryos harvested at the above defined time points were labeled by $^3$H-uridine (sp. act. 962 GBq/mmol; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) at a final concentration of 4 MBq/mmol (Laurincik et al., 2000) for 20 min in a gas-equilibrated culture medium. After incubation with the radioactive precursor, the specimens were repeatedly washed in $^3$H-uridine-free culture medium and fixed as described below.

### Processing for Light Microscopic Autoradiography and Transmission Electron Microscopy

After labelling with the radioactive precursor, the embryos were fixed in 3% glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.2). Subsequently, the specimens were washed in buffer, post-fixed in 1% $OsO_4$ in 0.1 M Na-phosphate buffer, embedded in Epon, and serially sectioned into semi-thin sections (2 µm). Every

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second section was stained with basic toluidine blue and evaluated by bright field light microscopy. Selected semi-thin sections were re-embedded according to Hyttel and Madsen (1987) and processed for ultra-thin sectioning (70 nm). The ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined on a Philips CM100 transmission electron microscope. Selected unstained semi-thin sections were processed for an autoradiography to detect a total RNA synthesis and nucleolus-associated RNA synthesis (Laurincik et al., 2000).

## RESULTS AND DISCUSSION

The processes taking place in the reconstructed embryos immediately after the fusion of the recipient ooplast (cytoplast) and the donor nucleus (karyoplast) are still poorly understood. Information on the mechanism accompanying chromatin remodelling could help to increase the success of the production of cloned animals. A nature of the oocyte-derived factors responsible for reprogramming is largely unknown, although it is clear from activation studies that their existence is transitory. From the point of view of the normally fertilized oocyte, their limited persistence is undoubtedly sufficient for the task of rapidly demethylating the incoming paternal DNA, but the highly differentiated state of transplanted somatic donor karyoplast may be more problematic (Armstrong, 2006).

In the present study, a nuclear transfer (SCNT) was followed by a period, when chromosomes were densely packed and thus transcriptionally inactive (Fig.1, B). From 1-2 hr post activation (hpa), a gradual

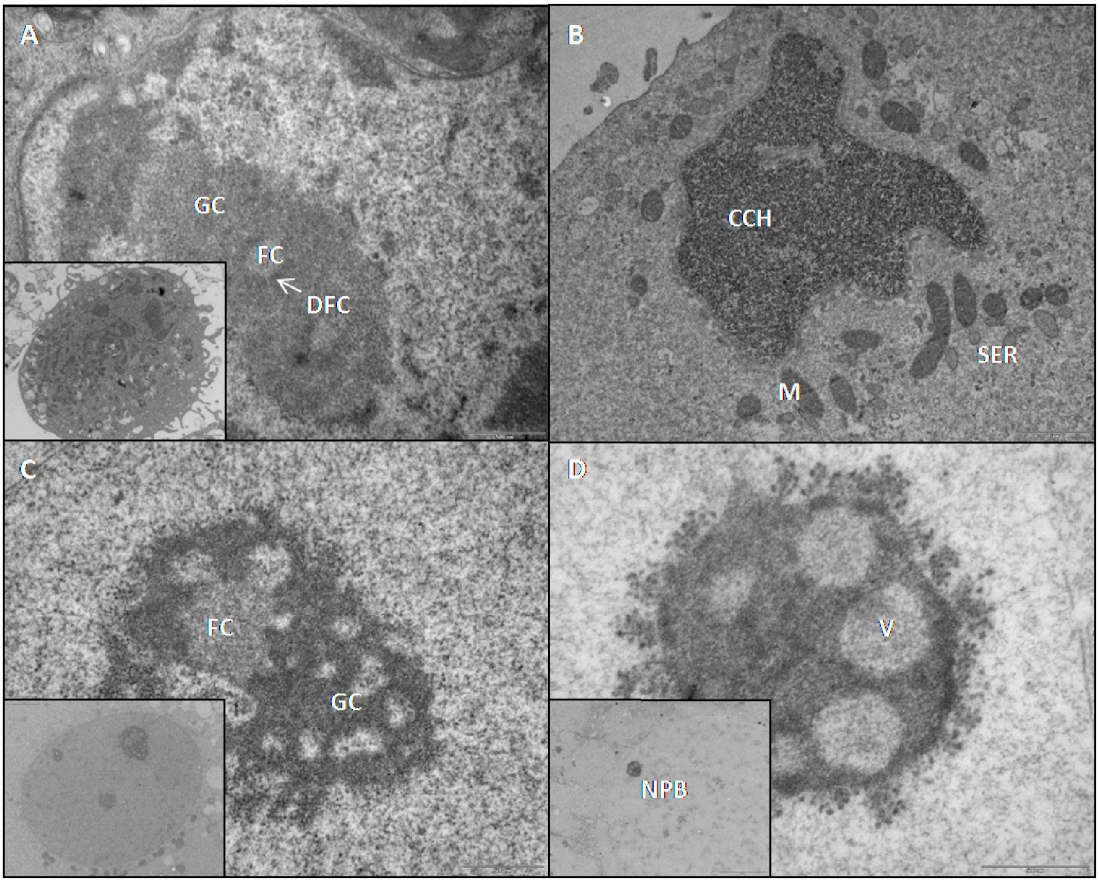


**Fig. 1: Transmission electron micrographs of fibroblast (A) and somatic cell nuclear transfer (SCNT) bovine embryos at 0.5 hpa (B), 4 hpa (C), 12 hpa (D)**

**A-** Starved fibroblast with fibrillo-granular nucleoli with fibrillar centres (FC), dense fibrillar component (DFC) and granular component (GC); B- Condensed chromatin (CCH) of the donor cell at 0.5 hpa closely surrounded by somatic cell cytoplasm, as indicated by somatic mitochondria (M) and smooth endoplasmic reticulum (SER); C- Pronucleus-like nucleus in SCNT embryo at 4 hpa displaying nucleolar structures with fibrillar centres (FC) and granular component (GC); D- SCNT embryo at 12 hpa with nucleolus precursor bodies (NPBs) displaying multiple peripheral vacuoles (V), surrounded by large electron-dense granules associated with condensed chromatin

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chromatin decondensation appeared and nuclei displayed large euchromatic and sparse heterochromatic area. In our experiments we did not observe nuclear envelope breakdown (NEBD) and chromatin condensation (CCA) in five embryos from 1.5-3 hpa when nuclei from fibroblasts, presumably arrested in GO/G1, were introduced into MII cytoplasm. These results are similar to those of Dominko et al. (1999) and Ouhibi et al. (1996). Many factors may explain these results. First, all nuclei that are transferred into the cytoplast with a high MPF activity undergo nuclear envelope breakdown and chromosome condensation, however MPF is predominantly associated with the spindle, and the enucleation will decrease its activity. This logically decreases CCA activity too (Fulka et al., 2001). On the other hand, the fact that the somatic cytoplast was introduced together with a somatic nucleus into the non-activated bovine cytoplast, as indicated by increased population of somatic mitochondria around the reforming nuclei during the 0.5-3 hpa (Fig. 1, B), may be taken as an indication that this process of remodelling is a sequential process depending on cytoplasmic factors from somatic cell and on energy supply from the somatic cell. This feature may hamper the import of ooplasmic factors to the somatic cell nucleus during this period which, at least from the partial lack of the nuclear envelope, may represent a particularly permissive period for the deprogramming. However, the presence of a spherical pronucleus-like nucleus and similar NPB-like structures in SCNT embryos from 4 hpa (Fig. 1, C) suggest that nuclear deprogramming occurs within these first hours after activation (Petrovicova et al., 2008).

Our ultrastructural study renews previous electron-microscopy observations of Lavoir et al. (1997), Kanka et al. (1999) and Baran et al. (2002) describing nucleolar changes in embryonic and somatic NT embryos during the first cleavage stages. The first nucleolus-related structures were observed in SCNT embryos at 1.5 hpa and only in the nuclei with a complete nuclear envelope. At 1.5 to 4 hpa, the nucleolus-related structures appeared either as bodies including large fibrillar centres and a granular component, but no dense fibrillar component, or as electron-dense spheres, i.e. nucleolus precursor bodies (NPBs) (Fig. 1, C). The most prominent nuclear entities in the remaining embryos at 4, 8, 12, and 16 hpa were NPBs. Interestingly, at 12 hpa, NPBs displayed multiple peripheral vacuoles (Fig. 1, D) whereas at 16 hpa the NPBs lacked of vacuolization. This sequential remodelling can be interpreted by a presence of the factors from the somatic cytoplast after 4 hpa by oocyte-derived factors (Petrovicova et al., 2008)

As has been demonstrated in previous studies, nucleolar transformation is associated with the first detectable embryonic RNA synthesis as shown by an incorporation of $^{3}$H- uridine. Thus, there is a clear correlation between nucleologenesis and nuclear RNA synthesis (Kopecny et al., 1989; Hyttel et al., 1998; Laurincik et al., 2000). Our observations showed that RNA synthesis was readily detected in starved bovine fibroblast before fusion (Fig.1, A). However, in the nuclear transfer reconstructed embryos, whatever the morphological appearance of the nucleoli, as previously described, no RNA was detected. These results clearly show that in reconstructed embryos the structural transformation of the nucleolus after fusion was not accompanied by the incorporation of $^{3}$H- uridine, and, therefore, nucleolar function was apparently disturbed.

In conclusion, mechanism, by which foreign nuclei are remodelled in the enucleated oocytes cytoplasm and whether the introduced nucleus is fully reprogrammed, is still not elucidated. However, basing on general appearance and the morphology of the nucleus, as well as the presence of nucleolus precursors bodies, we suppose that the process of remodelling is completed just at 4 hpa. In later time points the overall morphology was similar to the nucleus seen in pronuclei and, therefore, we assume that by this time, the nucleolus finally comes under the control of the factors contained in the ooplasm.

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## REFERENCES

ARMSTRONG, L. – LAKO, M. – DEAN, W. – STOJKOVIC, M. 2006. Epigenetic Modification Is Central to Genome Reprogramming in Somatic Cell Nuclear Transfer. *Stem Cells,* vol. 24, 2006, p. 805-814.

BARAN, V. – VIGNON, X. – LEBOURHIS,D. – RENARD, J. P. – FLÉCHON, J. E. 2002. Nucleolar Changes in Bovine Nucleotransferred Embryos. *Biology of Reproduction,* vol. 66, 2002, p.534-543.

CAMPBELL, K. H. S. – LOI, P. – OTAEGUI, P. I. – WILMUT, I. 1996. Cell cycle co-ordination in embryo cloning by nuclear transfer. *Reviews of Reproduction,* vol. 1, 1996, p.40-46.

DOMINKO, T. – MITALIPOVA, M. – HALEY, B. – BEYHAN, Z. – MEMILI, E. – MCKUSICK, B. – FIRST, N. L. 1999. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. *Biology of Reproduction,* vol. 60, 1999. p.1496-1502.

FULKA, J. jr. – LOI, P. – LEDDA, S. – MOOR, R. M. – FULKA, J. 2001. Nucleus transfer in mammals: how the oocyte cytoplasm modifies the transferred nucleus. *Theriogenology,* vol. 55, 2001, p. 1373-1380.

HYTTEL, P.- MADSEN, I. (1987): Rapid method to prepare mammalian oocytes and embryos for transmission electron microscopy. *Acta Anat.*, Basel vol. 129, p.12-14.

HYTTEL, P. – VIUFF, J. – LAURINČÍK, J. – AVERY, B. – KING, W. A. – FAIR, S. D. – THOMSEN, S. D. - HAY-SCHMIDT, A. – VAJTA, G. – CALLESEN, H. – OCHS, R. L. - GREVE T. 1998. Ribosomal RNA gene activation in pre-implantation bovine embryos. *Gametes: Development and function*. Serona Symposia Rome, 1998, p. 417-438. CHEN, N. – LIOW, S. L. – YIP, W. Y. – TAN, L. G. – TONG, G. Q. – NG, S. C. 2006. Dynamic changes in microtubules and early development of reconstructed embryos after somatic cell nuclear transfer in a non-human primate. *Cloning Stem Cells,* vol. 8, no. 4, 2006, p.251-258.

KANKA, J. – SMITH, S. D. – SOLOY, E. – HOLM, P – CALLESEN, H.1999: Nucleolar Ultrastructure in Bovine Nuclear Transfer Embryos. *Molecular Reproduction and Development*, vol. 52, 1999, p. 253-263.

KIM, J. M – OGURA, A. – NAGATA, M. – AOKI, F. 2002. Analysis of the mechanism for chromatin remodeling in embryos reconstructed by somatic nuclear transfer. *Biology of Reproduction,* vol. 67, 2002, p. 760-766.

KOPECNY, V. - FLÉCHON, J. E. - CAMOUS, S. - FULKA, J. jr. 1989: Nucleogenesis and the onset of transcription in the 8-cell bovine embryo: fine structural autoradiogradic study. *Mol. Reprod. Develop.*, vol. 1, 1989, p. 79-90.

LAURINČÍK, J. – THOMSEN, S. D. – HAY-SCHMIDT, A. – AVERY, B. – GREVE, T. – OCHS, L. R. – HYTTEL, P. 2000. Nucleolar proteins and nuclear structure in bovine embryos obtained *in vitro*. *Biol. Reprod.,* vol. 62, 2000, p. 1024-1032.

LAVOIR, M. C. – KELK, D. – RUMPH, N. – BARNES, F. – BETTERIGE, K. J. – KING, W. A.1997. Transcription and translation in bovine transfer embryos. *Biol Reprod.,* vol. 57, 1997, p. 204-213.

MISTELI, T. 2003. A nucleolar disappearing act in somatic cloning. *Nat Cell Biol*., vol. 5, no. 3, 2003, s.183-184.

OUHIBI, N. – FULKA, J. – KANKA, J. – MOOR, R. M. 1996. Nuclear transplantation of ectodermal cells in pig oocytes—ultrastructure and radiography. *Mol Reprod Dev.,* vol. 44, 1996, p. 533-539.

PETROVIČOVÁ, I. – ŠVARCOVÁ, O. – STREJČEK, F. – LUCAS-HAHN, A. – LEMME, E. – NIEMANN, H. – LAURINČÍK, J. – MADDOX-HYTTEL, P. 2008. Early aspect of uclear reprogramming following bovine somatic cell nuclear transfer. *Reproduction, fertility and development*, vol. 20, no.1, 2008, p. 105.

SZOLLOSI, S. – CZOLAWSKA, R. – SZOLLOISI, M. S. – TARKOWSKI, A. K. 1988. Remodelling of mouse thymocyte nuclei depends on the time of their transfer into activated, homologous oocytes. *J Cell Biol.*, vol. 91, 1988, p. 603-613.

WACHTLER, F. – STAHL, A. 1993. The nucleolus: A structural and functional interpretation. *Micron,* vol. 24, 1993, p. 473-505.

WRENZYCKI, C. – LUCAS-HAHN, A. – HERRMANN, D. – LEMME, E. – KORSAWE, K. – NIEMANN, H. 2002. *In vitro* production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. *Biol Reprod.,* vol. 66, p. 127-134.